

**EVALUATION OF SALMON SPERM ELECTROPORATION
FOR GENE TRANSFER.**

A thesis
submitted in fulfilment
of the requirements for the degree
of
Doctor of Philosophy
in the
Department of Zoology

by

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1997

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Abstract.

The aim of this thesis was to evaluate the potential of using sperm cells as a vector for gene transfer in chinook salmon (*Oncorhynchus tshawytscha*). Preliminary studies indicated that while salmon sperm were inefficient at picking up DNA during simple incubation, electroporation of the sperm significantly enhanced the sperm/DNA association. Therefore, a number of factors were examined to identify the optimal conditions for DNA uptake by salmon sperm and the subsequent transfer of foreign DNA into salmon embryos through fertilisation.

The effect of electroporation on salmon sperm/DNA association was examined using bacterial transformation, the polymerase chain reaction, dot blot and Southern analysis. Sperm were electroporated in the presence of circular or linear pRSV-lacZ DNA at 20 µg/ml and 100 µg/ml, with field strengths of 625 V/cm or 1000 V/cm, a pulse length of 27.4 ms, and with either a single or double pulse. Electroporation enhanced sperm retention of circular and linear pRSV-lacZ DNA, and retention was greatest at the higher field strength with two pulses, and a DNA concentration of 100 µg/ml. The sperm/DNA association was strong as it resisted repeated washing and digestion by DNase I, suggesting that some of the foreign DNA was internalised into the sperm. It was estimated that, following washing and DNase I digestion, on average, 0.12 fg (16 copies), of DNA were associated with each sperm cell.

The ability of electroporated sperm to transfer plasmid DNA into embryos was investigated using a number of combinations of field strength, pulse length, pulse number and DNA concentration. Optimal conditions were found to be two pulses of 27.4 ms at 1000 V/cm, using $0.5 \times$ HBS, with 200 µg/ml of plasmid DNA. The transferred DNA was detected by PCR in up to 85% of the resulting 12 week old fry. Gene transfer efficiencies of up to 47% were repeatedly achieved over two separate spawning seasons. Southern analysis failed to detect any plasmid DNA in these fry, indicating that although electroporated sperm are capable of transferring the plasmid DNA, the levels of this DNA in the fry are low and the resulting fry are mosaic.

PCR and Southern analysis of DNA extracted from embryos of five developmental stages was also carried out. Introduced plasmid DNA was detected in over 90% of the 0.8 day old embryos, and up to 10^7 copies of the plasmid DNA were observed in 1.2 day old embryos. The results indicated that the DNA introduced by electroporated sperm is amplified during the early stages of embryo development, remains predominantly extrachromosomal in an unmodified form, and is gradually lost during development. Expression of the introduced pRSV-lacZ DNA was detected in only a small number of embryos despite the presence of high levels of plasmid DNA in most of the embryos analysed.

The methods of sperm electroporation and egg microinjection for gene transfer were directly compared. In a parallel study, a growth hormone gene construct, OnMTGH1, was transferred into chinook and coho salmon using both these methods. While the microinjected DNA was converted into high molecular weight concatemers, the sperm transferred DNA remained unmodified after introduction into salmon embryos. A greater proportion of 11 day old embryos resulting from electroporated sperm (5/5) were found to contain OnMTGH1 DNA than those resulting from microinjected eggs (4/8). However, no introduced DNA, or growth enhanced individuals, were detected in the 10 month old salmon developing from sperm electroporated with the GH construct. In contrast, introduced DNA was detected in 12% of the microinjected group, and growth enhanced individuals were obtained.

It is clear from these results that electroporated salmon sperm can successfully take up DNA, and are efficient vectors for transferring genes into embryos. However, the introduced DNA does not persist and the levels decrease as the fish develop. Nevertheless, sperm electroporation has the potential to be further developed as an efficient mass gene transfer method in salmon.

Chapter One.

General Introduction.

Gene transfer has been accomplished in a variety of animals by the introduction of DNA into fertilised eggs. The first transgenic animal was produced by Gordon *et al.* (1980), using a glass micro needle to inject cloned DNA into the pronuclei of fertilised mouse eggs. The introduced DNA persisted and was passed on to the next generation. Following this initial study gene transfer was also successfully carried out in *Xenopus* (Rusconi and Schaffner, 1981; Etkin and Roberts, 1983), *Drosophila* (Spradling and Rubin, 1982), sea urchins (McMahon *et al.*, 1985), *Caenorhabditis elegans* (Stinchomb *et al.*, 1985) rabbits, pigs, sheep, cattle (Hammer *et al.*, 1985; Pursel *et al.*, 1989) and birds (Bosselman *et al.*, 1989).

To date there have been many studies involving gene transfer, and transgenic animals have been utilised for a range of purposes, including studies on development (Westpal and Gruss, 1985), DNA replication and gene expression (DePamphilis *et al.*, 1988), growth hormone function (Cioffi and Wagner, 1993), and nutrition (Knapp and Kopchick, 1994). Additionally transgenic mice are used as models for research on human illnesses such as cardiovascular disease (Sigmund, 1993), liver disease (Merlino, 1994), neurodegenerative diseases (Lee *et al.*, 1996) and breast cancer (Amundadottir *et al.*, 1996). Other applications of gene transfer technology include the manipulation of animals to create new strains with desired characteristics and the overproduction of useful proteins. For example, transgenic pigs with increased growth and improved feed conversion efficiencies have been produced (Pursel *et al.*, 1989), and human alpha-1-antitrypsin has been expressed in the milk of transgenic ewes (Carver *et al.*, 1993).

1.1 Gene transfer in fish.

There are two main reasons for the production of transgenic fish. Firstly, to increase the knowledge of gene regulation and development, and secondly, the transfer of economically important traits such as enhanced growth. Several characteristics of fish make them good candidates for transgenic studies. Compared to mammals they produce large numbers of relatively large eggs. Fertilisation and development in most species is

external, and development until hatching relies on maternal stores in the egg. The domestic culture and/or farming of some species means that gametes are readily available. Therefore the manipulations involved in gene transfer are more straightforward, and the cost of maintaining transgenic fish is lower than other transgenic animals.

The first successful attempt at producing transgenic fish was carried out by Zhu *et al.* (1985). In this study plasmid DNA containing the human growth hormone (GH) gene was microinjected into the blastodisc of fertilised gold fish (*Carassius auratus*) eggs and the introduced DNA was detected in fish up to 50 days old by DNA hybridisation analysis. To date gene transfer has been applied to a range of fish species, including Atlantic salmon (*Salmo salar*) (Fletcher *et al.*, 1988; Rokkones *et al.*, 1989), channel catfish (*Ictalurus punctatus*) (Hayat *et al.*, 1991), common carp (*Cyprinus carpio*) (Zhang *et al.*, 1990), loach (*Misgurnis fossilis*) (Kozlov *et al.*, 1988), medaka (*Oryzias latipes*) (Ozato *et al.*, 1986), northern pike (*Esox lucius*) (Gross *et al.*, 1992; Guise *et al.*, 1992), rainbow trout (*Oncorhynchus mykiss*) (Chourrout *et al.*, 1986; Maclean *et al.*, 1987a), tilapia (*Oreochromis niloticus*) (Brem *et al.*, 1988; Maclean *et al.*, 1992), walleye (*Stizostedion vitreum*) (Moav *et al.*, 1992), and zebrafish (*Brachydanio rerio*) (Stuart *et al.*, 1988).

Most early studies involved the transfer of mammalian genes and promoters, although a number of fish genes have now been isolated and several of these have been transferred into fish (Fletcher and Davies, 1991; Hackett, 1993; Gong and Hew, 1995). These include the rainbow trout and chinook salmon (*Oncorhynchus tshawytscha*) growth hormone (GH) cDNA clones (Zhang *et al.*, 1990; Du *et al.*, 1992a; Devlin *et al.*, 1995), the sockeye salmon (*O. nerka*) GH gene (Devlin *et al.*, 1994), and the rainbow trout insulin like growth factor (IGF) I cDNA clone (Chen *et al.*, 1995). Many other fish genes have also been isolated, including the chum salmon (*O. keta*) IGF I gene (Kavsan *et al.*, 1993), the carp α -globin gene (Yoshizaki *et al.*, 1991), and the prolactin genes of tilapia (*Oreochromis mossambicus*) (Swennen *et al.*, 1992) and chinook salmon (Xiong *et al.*, 1992). The number of fish genes available continues to increase and strategies such as gene tagging are being applied to this end (Gong *et al.*, 1994).

The promoter elements of several fish genes have also been isolated and shown to express in cultured fish cells or transgenic systems. These include promoters from the metallothionein genes of *Xiphophorous maculatus* (Fredenreich and Scharf, 1990;

Winkler *et al.*, 1991), rainbow trout (Zafarullah, *et al.*, 1988) and sockeye salmon (Chan and Devlin, 1993, Devlin *et al.*, 1994), the carp β -actin gene promoter (Liu *et al.*, 1990; Moav *et al.*, 1993), the antifreeze protein (AFP) promoters of ocean pout (*Macrozoarces americanus*), wolf fish (*Anarhichas lupus*), sea raven (*Hemittiperus americanus*) and winter flounder (*Pseudopleuronectes americanus*) (Gong *et al.*, 1991) and the histone H3 and protamine gene promoters of sockeye salmon (Chan and Devlin, 1993). When introducing genes into animals the use of homologous genetic elements is thought to result in more efficient expression. Several constructs have been assembled where all the components are of piscine origin (Du *et al.*, 1992a; Cavari *et al.*, 1993; Devlin *et al.*, 1994) and expression vectors based on promoter and terminator regions from fish genes have been developed (Liu *et al.*, 1990; Du *et al.*, 1992b; Caldovic and Hackett, 1995).

Fish are becoming increasingly important as models in biology (Powers, 1989) and species such as the zebrafish are well developed as systems for the study of vertebrate development (Rossant and Hopkins, 1992; Driever *et al.*, 1994). Gene transfer provides a powerful tool for the study of fish development and the determination of the role of different genes (Hackett, 1993; Gong and Hew, 1995). For example, the expression pattern driven by mouse homeobox promoters has been analysed in zebrafish (Westerfield *et al.*, 1992).

A major focus of the research on transgenic fish has been the transfer of GH genes. Gene constructs containing GH genes have been transferred into a range of species, including goldfish (Zhu *et al.*, 1985), rainbow trout (Chourrout *et al.*, 1986, Rokkones *et al.*, 1989; Penman *et al.*, 1990, Inoue *et al.*, 1993), Atlantic salmon (Rokkones *et al.*, 1989; Du *et al.*, 1992a), coho salmon (*Oncorhynchus kisutch*), chinook salmon, rainbow trout, cutthroat trout (*O. clarki*) (Devlin *et al.*, 1994; Devlin *et al.*, 1995), carp (Zhang *et al.*, 1990; Zhu, 1992; Chen *et al.*, 1993), tilapia (*Oreochromis niloticus*) (Brem *et al.*, 1988; Martinez *et al.*, 1996), northern pike (Gross *et al.*, 1992; Guise *et al.*, 1992), medaka (Lu *et al.*, 1992) and zebrafish (Zhao *et al.*, 1993). Although successful gene transfer resulted, in many of these studies there was either no enhancement of growth, or only a moderate elevation of the growth rate of the fish was obtained. This may in part be due to inefficient expression from gene constructs consisting of viral promoters and/or mammalian genes. In others studies where all fish gene constructs were used (opAFPGH or OnMTGH1) a six to

ten fold increase in growth was reported (Du *et al.*, 1992a; Devlin *et al.*, 1994; Devlin *et al.*, 1995).

Another application of fish transgenic technology is the transfer of antifreeze protein (AFP) genes, which if successful would enable fish to be farmed at lower temperatures. The complete winter flounder genomic AFP gene has been successfully transferred into Atlantic salmon (Fletcher *et al.*, 1988). Although transmission to progeny and expression of the introduced AFP gene has been demonstrated (Shears *et al.*, 1991), the level of the protein in the salmon is presently too low to confer freeze resistance and strategies such as increasing the copy number of the transgene or the use of alternative promoters may have to be applied to achieve this (Hew *et al.*, 1992). However, the ocean pout AFP (opAFP) gene was found to give increased cold tolerance when introduced into goldfish (Wang *et al.*, 1995).

It is also hoped that gene transfer may be used to increase the disease resistance of fish. There is evidence that lysozyme, a non-specific antibacterial enzyme, is important in salmonid disease resistance (Grinde, 1989). A rainbow trout lysozyme cDNA clone has been isolated (Dautigny *et al.*, 1991) and Hew *et al.* (1995) injected this cDNA clone, under the control of the opAFP promoter, into Atlantic salmon eggs. Analysis of the resulting fish has yet to be reported.

The production of sterile fish is seen as a possible application of gene transfer technology (Maclean and Penman, 1990; Devlin and Donaldson, 1992; Gong and Hew, 1995) and as a necessary requirement if transgenic fish are to be farmed. With the long term aim of using gonadotropin releasing hormone (GnRH) antisense RNA to induce sterility in fish, Husebye *et al.* (1995) demonstrated that a reporter construct, under the control of a salmon GnRH gene promoter, was specifically expressed in the hypothalamus when transferred into zebrafish. Thus indicating that such a method is a feasible approach for producing sterile fish.

1.2 Gene transfer techniques.

1.2.1 Microinjection.

Out of the several methods applicable to the genetic manipulation of fish, direct introduction of DNA into fertilised zygotes by microinjection is the most commonly

used technique (for reviews see: Fletcher and Davies, 1991; Hackett, 1993; Gong and Hew, 1995). The method is similar to the method used for gene transfer in mice where a glass needle is used to inject DNA into the male pronucleus of a fertilised 1 cell embryo (Gordon *et al.*, 1980; Gordon, 1994). However, there are several difficulties involved in applying this technique to fish due to the toughness and/or opaqueness of the chorion of fish eggs and the inability to visualise nuclear structures. Therefore the foreign DNA is normally injected into the cytoplasm of fertilised eggs, either prior to pronuclear fusion (for example, Fletcher *et al.*, 1988), at the early 1 cell stage (for example, Penman *et al.*, 1990), the late 1 cell stage (for example, Guyomard *et al.*, 1989), or between the 1 and 4 cell stage (for example, Stuart *et al.*, 1988; Chong and Vielkind, 1989). While cytoplasmic injection is the most common method, injection into the pronucleus of medaka oocytes has been carried out successfully (Ozato *et al.*, 1986; Inoue and Yamashita *et al.*, 1993).

To avoid the difficulties involved in fish egg injection, various injection strategies and procedures have been developed depending on the species of fish and the experience of the individual (Fletcher and Davies, 1991). For example, most salmonid eggs have a tough chorion which is difficult to penetrate during injection, making it necessary to devise alternative injection strategies, such as injection through the micropyle (Fletcher *et al.*, 1988), the use of a larger, sturdier needle (Maclean *et al.*, 1987a; Maclean, 1993), and cutting a small hole in the chorion (Rokkones, *et al.*, 1989; Chourrout *et al.*, 1986). Microinjection has also been assisted by the removal of the chorion by trypsin digestion of goldfish eggs (Zhu *et al.*, 1985), or manual removal from zebrafish eggs (Stuart *et al.*, 1988).

A disadvantage associated with the use of microinjection for gene transfer in fish is that majority of the resulting fish appear to be mosaic and it seems that the DNA introduced into fish integrates at the two cell stage or later (Fletcher and Davies, 1991; Hackett, 1993). While this may be due to the cytoplasmic nature of the injection procedures utilised in most studies, mosaic fish are also produced when the DNA is injected into the oocyte pronucleus (Ozato *et al.*, 1986)

Gene transfer frequencies in fish generated by microinjection vary from 3 to 50% (Brem *et al.*, 1988; Guyomard *et al.*, 1989). Typically higher proportions of fish are

reported to contain introduced DNA when early developmental stages are examined, but this is not maintained, and when later stages are analysed, gene transfer rates are lower. The introduced DNA often fails to persist and the level of expression and/or proportion of fish containing the transgenes decrease as development continues (Fletcher and Davies, 1991; Hackett, 1993).

The microinjection technique is also problematic as it is a laborious, technically difficult, and time consuming procedure, and the number of potential transgenics produced is limited. Injection rates vary from between 50 to 1000 eggs per hour (Hackett, 1993). This is particularly a problem in seasonal species where gametes may be available for a only brief period. Although gametes may be stored for later use this is usually only possible for a few days and the quality of the eggs, an essential parameter for survival of injected eggs, may suffer.

1.2.2 Mass gene transfer methods.

Given the problems with microinjection it is hoped this method will be replaced in the future by a more effective system such as a mass gene transfer technique (Fletcher and Davies, 1991; Chen *et al.*, 1993; Hackett, 1993). The application of a mass gene transfer technique to fish is an attractive proposition because fertilisation is external and large numbers of individuals can be cultured in a relatively small space. The main criteria for the development of a mass gene transfer technique are that the method should be simple and reliable, enable the treatment of many embryos in a short time span, and produce a large number of viable transgenic individuals. Several methods have been developed in fish to transfer DNA into more than one egg at a time. These include embryo electroporation, microprojectile bombardment, lipofection and sperm transfer.

1.2.2.1 Embryo electroporation.

Embryo electroporation involves the application of an electric shock to fertilised eggs in the presence of DNA, and allows the uptake of DNA by enhancing permeability of the egg. Successful gene transfer using electroporation of fertilised fish eggs was first reported in loach by Xie *et al.* (1989). In this study a gene transfer rate of 10% was obtained following the exposure of dechorionated loach eggs to an exponentially

decaying electrical pulse of $500 \text{ V/cm} \times 5.1 \text{ ms}$, using a capacitor based electroporation apparatus. In a later study gene transfer efficiencies of up to 62.5% were obtained when dechorionated fertilised loach eggs were exposed to a single pulse of $600 \text{ V/cm} \times 0.9 \text{ ms}$ (Xie *et al.*, 1993).

Inoue *et al.* (1990) reported the successful introduction of DNA into non-dechorionated medaka embryos exposed to five exponentially decaying pulses of $750 \text{ V/cm} \times 50 \mu\text{s}$. Four percent of the surviving hatchlings contained the introduced DNA and evidence of germline transmission of the transgene was reported (Inoue *et al.*, 1990). A higher gene transfer efficiency of 65% was obtained when exponentially decaying electrical pulses were used to transfer DNA into zebrafish embryos (Buono and Linser, 1992). This study involved exposing non-dechorionated one- to two-cell zebrafish embryos to 3 pulses of $125 \text{ V/cm} \times 0.1 \text{ ms}$. While some attempts by other researchers to replicate these results met with success, others did not result in successful gene transfer, and it was suggested that the essential component of the procedure necessary for permeabilisation of the chorion was washing the embryos with the bactericide Wescodyne (Buono and Linser, 1992; Hackett, 1993). The level of mortality in the above studies was relatively high, from 25 to 32%, but this was offset by the fact that many eggs were treated in a short period relative to microinjection procedures. Lower mortality rates (10 to 28%) and higher gene transfer efficiencies (70 to 75%) were reported when Baekonization of non-dechorionated zebrafish eggs was assessed by Powers *et al.* (1992) and Zhao *et al.* (1993). Baekonization is based on a non-capacitor electroporation apparatus that can deliver a high number (up to 2^9) of high voltage square shaped electrical pulses, of relatively low current over a short period. This type of electroporation has also been successfully used to transfer DNA into the non-dechorionated fertilised eggs of common carp, channel catfish (Powers *et al.*, 1992), and black porgy (*Acanthopagrus schlegelii*) (Tsai and Tseng, 1994). Square wave electric pulses have also been demonstrated to be effective at introducing DNA into the dechorionated fertilised eggs of zebrafish, African catfish (*Claris gariepinus*) and rosy barb (*Barbus conchoni*) (Müller *et al.*, 1993). Thus electroporation of fish embryos appears to be reproducible and has been successfully applied to a number of species.

1.2.2.2 Microprojectile bombardment of embryos.

The technique of delivering foreign genes into cells using high-velocity microprojectiles coated with DNA has been used to transform plant (Klein, *et al.*, 1988; Maliga, 1993) and animal cells (Zelenin *et al.*, 1989; Fitzpatrick-McElligot, 1992; Jiao *et al.*, 1993). Gene transfer into chickens has been demonstrated following the bombardment of primordial germ cells with tungsten particles coated with plasmid DNA (Ying *et al.*, 1995). The hatchlings developing from the bombarded embryos were shown to contain the introduced DNA in their sperm and to pass it on to their progeny. However, it appeared from Southern analysis and the gradual disappearance of the DNA throughout development, that the introduced DNA remained unintegrated (Ying *et al.*, 1995).

Successful transfer of a gene construct containing a luciferase reporter gene by microprojectile bombardment into the embryos of *Artemia franciscana* was reported by Gendreau *et al.* (1995). Only transient expression of the introduced DNA was detected in this study, with no luciferase activity being detected 48 hours after bombardment. Transient expression of a reporter gene following microprojectile bombardment has also been demonstrated in sea urchin embryos (Akaska *et al.*, 1995). However, the amount of introduced DNA detected in the embryos was found to be low and inconsistent between replicates (Akaska *et al.*, 1995).

Zelenin *et al.* (1991) applied this method to the fertilised eggs of loach, rainbow trout and zebrafish. The eggs were bombarded with high-velocity tungsten microprojectiles covered with plasmid DNA and three days later a survival rate of 70% was obtained in all three species. Expression of the introduced DNA was detected in 5% of the three day old loach embryos. The presence of the introduced DNA was also demonstrated by PCR in 9 day old zebrafish alevins. However, as there was no analysis past this point, no conclusions can be drawn about the persistence of the DNA transferred into fish via this technique. Therefore, while microprojectile bombardment offers a viable method of treating many eggs in a short period, no evidence has been obtained to indicate that the DNA transferred by this technique persists or is integrated into the host genomic DNA.

1.2.2.3 Lipofection of embryos.

Lipofection involves encapsulating DNA in synthetic lipid vesicles which then fuse with the plasma membrane and has been used to transfer genes into cultured cells (Felgner *et al.*, 1987; Behr *et al.*, 1989; Loeffler *et al.*, 1990; Barthel *et al.*, 1993; Staedel *et al.*, 1994), tissue cultures (Takeshita *et al.*, 1994) and chicken embryos (Rosenblum and Chen, 1995). This system has also been used to introduce recombinant bacteriophage lambda particles into African catfish embryos following dechorionisation (Szelei *et al.*, 1994). However, while the transfer efficiency was high, with expression of the introduced DNA in 60 to 80% of the six-day old larvae, no evidence of persistence of the DNA was found in four month old individuals.

1.2.2.4 Sperm transfer.

Several characteristics of fish make them suitable candidates for sperm-mediated gene transfer. For example, fish sperm are not motile in seminal fluid, and only become active after contact with water or ovarian fluid. External fertilisation and the ease of collection and storage of fish gametes also make the use of this technique suitable in fish.

The use of sperm for gene transfer was first reported in rabbits by Brackett *et al.* (1971). Over a decade later, successful gene transfer following incubation of DNA with the sperm of the sea urchin (Arezzo, 1989) and mouse was reported (Lavitrano *et al.*, 1989a). Although further experiments using mouse sperm immediately after this failed to replicate these results (Brinster, 1989; Lavitrano *et al.*, 1989b), the incubation of DNA with sperm has since been successfully employed to transfer genes into embryos from several species, including cattle (Perez, *et al.*, 1991; Sperandio, *et al.*, 1996), mice (Hochi *et al.*, 1990), pigs (Gandolfi *et al.*, 1989; Sperandio, *et al.*, 1996) and *Xenopus* (Habrova *et al.*, 1996).

Several studies examining the interaction of DNA with sperm have collected evidence that, following incubation, DNA is taken up by sperm and is internalised into the sperm heads. For example, after incubation with rabbit sperm, radiolabelled simian virus 40 (SV40) DNA was detected in the postacrosomal region of the sperm by autoradiography (Brackett *et al.*, 1971). Castro *et al.* (1990), reported the uptake of labelled DNA by mouse, rooster, boar, bull, buffalo, ram, goat and carp sperm. The association of the DNA with the sperm was strong, as radioactivity was still detected in sperm samples after 5 washes or digestion with DNase. Similarly, Atkinson *et al.* (1991)

found that a proportion of the DNA taken up by bull sperm remained after DNase digestion, implying that the DNA had been internalised into the sperm giving it protection from degradation. DNA incubated with pig sperm was also shown to become either tightly bound to, or internalised by pig sperm, as the sperm/DNA association resisted repeated washes, gradient centrifugations, and prolonged dialysis (Horan *et al.*, 1991). *In situ* hybridisation revealed that the DNA associated with the pig sperm was attached to the periphery of the sperm heads and around the nuclear ring, and not to the sperm tails. Additionally, when DNA was incubated with mouse, cattle, pig and human sperm, the bound DNA was detected over the equatorial and postacrosomal region of the sperm head, in the region corresponding to the location of the nucleus (Lavitrano *et al.*, 1992; Atkinson *et al.*, 1991; Camaioni *et al.*, 1992; Gandolfi, *et al.*, 1996). Francolini *et al.* (1993) examined the uptake of exogenous DNA by mouse sperm cells in greater detail. Following incubation with end-labelled DNA for 2 hours, isolation of nuclei and ultrastructural autoradiography indicated that the DNA had not only entered the sperm cells, but was internalised into 65% of the sperm nuclei. These results provide good evidence that after incubation the DNA becomes strongly associated with sperm, is internalised, and may in fact be entering the sperm nuclei.

The incubation of sperm with DNA has been applied to gene transfer in fish. When attempted by Chourrout and Perrot (1992) the transfer of linear plasmid DNA into embryos by incubation with rainbow trout sperm was unsuccessful. However, the rainbow trout sperm did appear to take up DNA, as the amount of DNA detected in solution after incubation decreased when the concentration of the sperm was increased. Zebrafish sperm were shown to be capable of taking up plasmid DNA and transferring this into zebrafish fry (Khoo *et al.*, 1992). Transfer efficiencies of up to 53% (8/15) and transmission to the F1 and F2 generations were reported. However, no expression from the transferred USVCAT gene construct was detected and much of the introduced DNA appeared to exist extrachromosomally.

Although there have been reports of detection of sperm transferred DNA in adult mice, chicken, pigs and fish, in many cases the presence of the introduced DNA is only observed in embryos (Lauria and Gandolfi, 1993). It seems that the introduced DNA is unstable, and is either lost during development, or highly mosaic animals are generated (Lauria and Gandolfi, 1993). Two methods that have been applied to try and improve sperm gene transfer are: (1) the use of liposomes and (2) electroporation of the sperm.

Bachiller *et al.* (1991) found that the packaging of DNA into liposomes appeared to enhance the uptake of DNA by mice sperm. Evidence was obtained that the liposome transfected DNA was internalised into the sperm and was located in or near the nucleus. However, no introduced DNA was detected in mice developing from eggs fertilised with such sperm (Bachiller *et al.*, 1991). The uptake of DNA by chicken sperm has also been shown to be enhanced by the encapsulation of plasmid DNA in liposomes (Rottmann *et al.*, 1992; Squires *et al.*, 1993). Additionally, sperm treated in this way were shown to be capable of transferring plasmid DNA into chicken embryos (Rottmann *et al.*, 1992). However, the introduced plasmid DNA detected by Southern analysis in this study was not integrated into the chicken genome. Nakanishi and Iritani (1993) also found the DNA uptake and transfer by chicken sperm was enhanced by the use of liposomes. The resulting gene transfer efficiencies were 63% (34/54) when liposomes were employed and 47% (14/30) when DNA was simply mixed with the sperm. It should be noted that only embryos from newly laid eggs were analysed and the relatively sensitive method of PCR was used for DNA detection. Rottmann *et al.* (1996) recently reported successful gene transfer into rabbits, cattle and chicken embryos using liposome-mediated sperm transfer. This method has only recently been attempted in fish. However, no evidence of gene transfer was detected following the incubation of liposome packaged DNA with coho salmon sperm (Robert Devlin, personal communication).

Electroporation has been found to increase DNA uptake by cattle, pig and chicken sperm (Gagnè *et al.*, 1991; Horan *et al.*, 1992; Nakanishi and Iritani, 1993). Additionally, DNA transfer by electroporated sperm was detected in cattle and chicken embryos, although there was no evidence that this was more efficient than sperm/DNA incubation on its own (Gagnè *et al.*, 1991; Nakanishi and Iritani, 1993).

In contrast electroporation has been shown to increase the efficiency of gene transfer by fish sperm. Gene transfer by electroporated sperm of common carp, African catfish and tilapia was reported by Müller *et al.* (1992), with 2.6 to 4.2% of the embryos analysed containing the introduced DNA. Additionally, the expression of the transferred DNA was demonstrated in African catfish fry. When non-electroporated sperm of these species were used no transfer was detected (Müller *et al.*, 1992). A preliminary study in our laboratory also demonstrated that electroporated chinook salmon sperm are capable of transferring DNA into embryos (Sin *et al.*, 1993). Up to 10% of the fry resulting from electroporated sperm were found to contain the introduced DNA while no transfer by

non-electroporated sperm was observed. Tsai *et al.* (1995) demonstrated that electroporated (Baekonized) loach sperm take up DNA and used this technique to transfer a salmon growth hormone gene construct into loach. Additionally, expression of the transferred opAFPGHc construct was indicated by the appearance of a growth enhanced phenotype in groups resulting from treated sperm (Tsai *et al.*, 1995). A more recent study (Patil and Khoo, 1996) indicated that sperm DNA transfer into zebrafish was enhanced by electroporation. Up to 14.5% of the two week old fry were found to contain introduced DNA when electroporated sperm were used, compared to 6.5% for non-electroporated sperm.

1.3 The use of sperm and electroporated sperm for gene transfer in chinook salmon.

Chinook salmon are a major aquacultural species in New Zealand and are farmed in fresh and salt-water. With the long term goal of using transgenic technology to improve economically important traits of chinook salmon, such as growth rate, it was necessary to develop an efficient gene transfer method in this species. Other experimental fish species, with shorter generation times and greater access to gametes, could have been employed in the initial development of this technique. However, the number of alternative species was limited to small warm water fish such as the zebrafish which produce only a small amount of sperm compared to salmon. The difference in the volume of semen, together with other species specific differences, would mean that a considerable amount of further optimisation would be required to subsequently apply the technique to chinook salmon. Therefore development of the sperm gene transfer technique was carried out directly in this species.

The choice of gene transfer technique was constrained by several factors. Firstly, the availability of sexually mature chinook salmon in New Zealand is limited to only 3 to 4 weeks of the year (May/June), and secondly salmon rearing could not be carried out at the University, but at a small hatchery with limited facilities. Therefore, a technique that was quick, relatively technically simple, and could be applied to many eggs at once was required. Given the technical difficulties of microinjecting salmon eggs, the slow rate of production of transgenics using this method, and the limitations of the hatchery, this method was not suitable. Egg electroporation cannot be applied to salmonids due to the size of the eggs and as the eggs have a hard chorion which cannot be easily removed.

As sperm transfer is easy to carry out and can be used to treat a large number of eggs in a short space of time, this method was selected to be evaluated as a gene transfer method in this species. At the time that the salmon sperm transfer study was initiated in 1990, there were no published reports of sperm DNA uptake or transfer by fish sperm, although successful transfer had been reported in rabbits (Brackett *et al.*, 1971), sea urchins (Arezzo, 1989) and mice (Lavitrano *et al.*, 1989a) using this method.

In addition to the direct incubation of sperm with DNA, sperm electroporation was employed to determine if this would increase DNA uptake and gene transfer efficiencies. Electroporation is the transient permeabilisation of membranes by the application of an electric field. The technique is based on the observations that when cells are exposed to an electric field a potential is induced across the plasma membrane, and when this reaches a critical value membrane breakdown occurs (Chang *et al.*, 1992). Neumann and Rosenheck (1972) showed that reversible non-destructive electroporation of cell membranes could be achieved by applying high electric field pulses of short-duration. Neumann *et al.* (1982) used this process to introduce the herpes simplex virus into cultured mouse lymphoma cells. Since this first application of electroporation for gene transfer, this method has successfully been used for gene transfer in a variety of cells (Potter *et al.*, 1984; Chu *et al.*, 1987; Shigekawa and Dower, 1988; Andreason and Evans, 1988; Förster and Neumann, 1989; Orlowski and Mir, 1993).

The theory of the mechanism behind this electrically induced poration was described by Weaver (1993; 1995). The reversible permeability is thought to occur because, as the cell membrane acts as an electrical capacitor through which current is unable to pass, local areas of breakdown occur followed by the formation of hydrophilic pores, which subsequently reseal (Weaver, 1993; 1995). When the transmembrane voltage reaches 0.5 to 1 V, well above the normal resting potential of approximately 0.1 V, a high conductance state, known as reversible electrical breakdown (REB) occurs (Weaver, 1995). This breakdown involves a rapid membrane discharge in which the transmembrane voltage returns to low levels a few microseconds after the pulse. However, complete membrane recovery does not occur for some seconds to minutes after this. The conditions that cause REB are the same as those associated with molecular transport into cells. The actual mechanism of the pore formation is thought to rely on rapid structural rearrangements within the membrane (Weaver *et al.*, 1995).

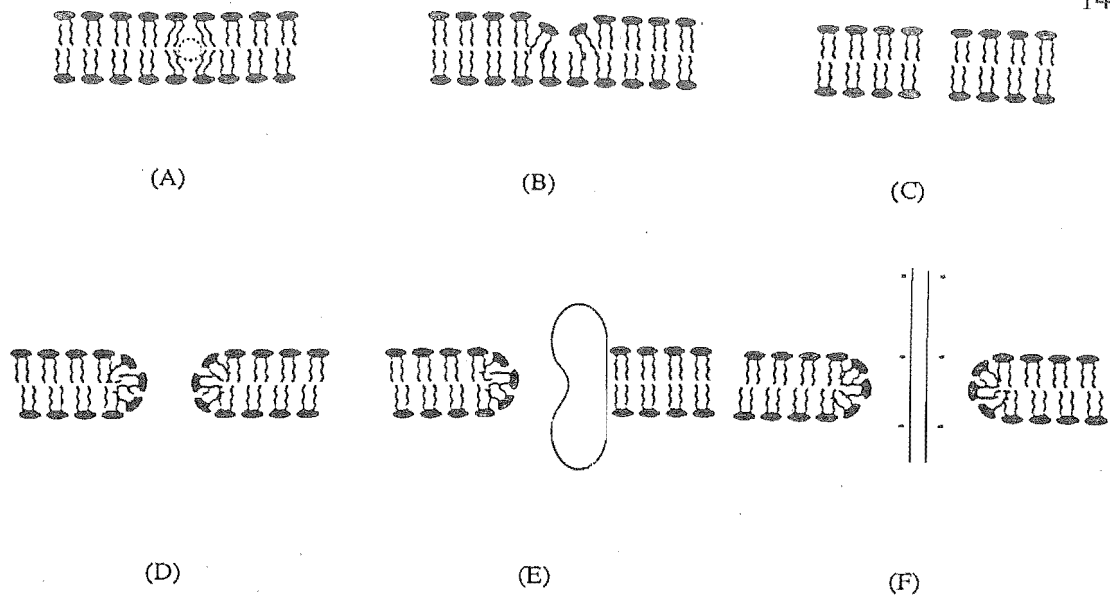


Figure 1.1. Drawings of hypothetical membrane structures involved in electroporation. (A) Free volume fluctuation. (B) Aqueous protrusion or "dimple". (C) Hydrophobic pore. (D) Hydrophilic pore, regarded as the "primary pores" through which ions and molecules pass. (E) Composite pore with protein at the pore's inner edge. (F) Composite pore with a charged macromolecule inserted into a hydrophilic pore. Reproduced from Weaver *et al.* (1993).

Figure 1.1 (A-D) shows the theoretical sequence of events involved in electroporation. The onset of pore formation is thought to be initiated through an interaction between thermal fluctuations and an elevated transmembrane voltage. Hydrophobic pores are thought to form first (Figure 1.1C), as less energy is required for their formation (Weaver, 1993). The hydrophilic pores (Figure 1.1D), that are presumably active in the molecular transport, then form. The hydrophilic pores may be involved in interactions with transmembrane proteins (Figure 1.1E), other large charged molecules attached to the cell, or molecules being transported into the cell (Figure 1.1F), all of which could have a stabilising effect prolonging the lifetime of the pore. The hydrophilic pores involved in transport across the membrane are likely to be quite small, in the range of 1 to 10 nm in diameter (Sukharev *et al.*, 1992) and fluctuate in size and position (Barnett and Weaver, 1991). Much larger secondary pores may form as primary pores are enlarged by pressure driven flows (Weaver, 1993). Pore like structures, 20-120 nm in diameter, have been visualised by freeze-fracture electron microscopy following electroporation of red blood cells (Chang and Reese, 1990) and are thought to be such secondary pores (Weaver, 1993).

The mechanism of the passage of DNA transfer across the membrane following electroporation is not well understood. Theories include direct diffusion of DNA through the pores, DNA binding to the membrane followed by lateral diffusion through the pores, and the electrophoretic transport of the DNA across the membrane (Xie *et al.*, 1990; Dimitrov and Sowers, 1990). In a study carried out by Sukharev *et al.* (1992), evidence was obtained that the DNA uptake is driven by an electrophoretic process. The results suggested that the DNA may increase the size of the pore as it is drawn through the membrane by the electrophoretic force. This process could allow a DNA molecule larger than the size of the original pore to enter the cell. Additionally, the presence of the pores may be extended as expanded pores may remain open for longer, and the DNA molecule may prevent resealing if it is part way through a pore when the electric field is turned off.

Electroporation has several advantages over other methods used for gene transfer. Large DNA molecules, up to 150kb (Shigekawa and Dower, 1988), can be introduced by this method, whereas size may be a limitation with other methods, for example, retroviral gene transfer (Eglitis and Anderson, 1988; Jaenisch, 1988; Potter, 1993). In addition, a lower mutation rate has been observed in DNA transferred by electroporation compared to alternative methods, such as calcium phosphate or DEAE-dextran facilitated transfer (Potter *et al.*, 1993). In these methods the DNA gains entry into the cell via endocytosis as it is incorporated into phagocytic vesicles, whereas the DNA introduced upon electroporation is essentially naked. Electroporation is also relatively quick and easy to carry out, and typically results in high yields of transformation, (up to 60 to 80%) (Förster and Neumann, 1989; Chang *et al.*, 1992). It is less damaging to the target cells and transfected DNA than alternative methods and, as it relies on a physical rather than a chemical process, it can be applied to a wide range of cell types (Chang *et al.*, 1992; Potter, 1993).

Factors found to influence gene transfer efficiency by electroporation are either electrical or biological in nature. Electrical parameters include the strength of the electric field, the pulse length and the shape of the electric pulse. Two main types of electrical pulse have been used to electroporate cells: exponential decay and square wave. The voltage of exponential decay pulses, delivered by capacitor discharge, is raised to an initial peak amplitude then decays exponentially, whereas the same voltage is maintained for the duration of a square wave pulse. Many studies on gene transfer by electroporation have

employed exponential decay pulses because of its relative ease and low costs (Förster and Neumann, 1989). Electric fields oscillating at a radio frequency have also been used for electroporation (Chang, 1989; Chang, 1992). This method results in an increased viability of the treated cells, but more complicated and expensive equipment is required (Chang, 1992).

The field strength and pulse length are the most important electrical variables affecting the efficiency of gene transfer by electroporation (Shigekawa and Dower, 1988; Chang, 1992). The strength of the electric field applied is a function of the voltage used and the distance between the electrodes. This factor has a large influence on the efficiency of electroporation. Low transfection rates are gained at low field strengths, then increase sharply at a certain threshold level, and decrease again after reaching a maximum, as the field strength is increased further (Andreason and Evans, 1988; Shigekawa and Dower, 1988). The length of a square electric pulse is set by the delivery device (Chang, 1992). The length of an exponentially decaying pulse is also determined by the apparatus used, by the capacitance setting, but is also influenced by the resistance of the sample, higher resistances producing longer pulses. The length of the pulse and the field strength have a complementary relationship. If the length of the pulse is relatively short, a high field strength is necessary to obtain satisfactory gene transfer efficiencies, but if a longer pulse is used then the required field strength is much lower (Shigekawa and Dower, 1988; Chang, 1992). The level of cell viability decreases as the strength of the electric field and the length of the pulse increase (Chu *et al.*, 1987; Andreason and Evans, 1988; Shigekawa and Dower, 1988). As there is only a general correlation between cell death and maximum transfection efficiency the optimal combination of field strength and pulse length must be experimentally determined for each cell type (Shigekawa and Dower, 1988).

Other factors that have been shown to influence the efficiency of gene transfer by electroporation include the composition of the buffer, the DNA concentration, the form and size of the DNA, and the addition of carrier DNA. The response of different cells to any of these variables cannot be predicted with certainty, and the conditions required for efficient gene transfer into a particular cell type using electroporation need to be experimentally determined (Shigekawa and Dower, 1988; Chang, 1992).

1.4 Aims.

In 1990 a preliminary study was carried out to determine if electroporated salmon sperm could be used for gene transfer (Sin *et al.*, 1993). While this study demonstrated the feasibility of using this method, the optimal conditions for gene transfer by electroporated salmon sperm were not determined. Therefore the main aims of this thesis were:

- (1) to further evaluate gene transfer into chinook salmon using sperm, and to determine if electroporation increases the efficiency of DNA uptake and gene transfer,
- (2) to determine the optimal conditions for gene transfer by electroporated chinook salmon sperm,
- (3) to examine the fate and expression of the DNA introduced into salmon by sperm,
- (4) to compare the efficiency of gene transfer using sperm with transfer by egg microinjection and,
- (5) to determine whether the optimised sperm electroporation technique could be used to produce growth enhanced chinook and coho salmon.

Chapter 2 describes the assessment of DNA uptake by salmon sperm. The effect of the electrical parameters of the pulses, the field strength and pulse length, and the concentration and form of the DNA, on this uptake was evaluated. The effect of these parameters, as well as the number of electrical pulses applied, on gene transfer into salmon embryos and fry was also analysed, and these results are presented in Chapter 3. Chapter 4 outlines the examination of the fate of the DNA introduced into salmon embryos by sperm, and addresses factors such as the amount, fate and ability of the DNA to be expressed. In order to further evaluate the use of sperm for gene transfer, and to directly compare this to egg microinjection, a growth hormone gene construct known to enhance the growth of pacific salmon was used. The results, including the transfer efficiency into embryos, and the amount, fate and persistence of the introduced DNA, are presented in Chapter 5. Chapter 6 contains a general discussion of the data presented in this thesis.

Chapter Two.

DNA uptake by salmon sperm.

2.1 Introduction.

The uptake of DNA by sperm and the subsequent passage of genes into embryos during fertilisation is an attractive alternative technique for the production of transgenic animals. In mammals this would avoid the time consuming, difficult and often deleterious procedures of removing, injecting and reimplanting eggs. In fish it would provide a potential mass gene transfer technique as treated sperm could be used to fertilise many eggs in a single application.

The first study reporting uptake and transfer of DNA by sperm was carried out by Brackett *et al.* (1971). After incubation with rabbit sperm, radiolabelled simian virus 40 (SV40) DNA was detected in the postacrosomal region of the sperm by autoradiography. The foreign DNA also appeared to be transferred to rabbit ova through fertilisation as infectious SV40 was recovered when CV-1 cells were fused with 2 cell embryos (Brackett *et al.*, 1971). Sea urchin sperm have also been shown to be capable of taking up radioactively labelled DNA (Arezzo, 1989). Additionally, based on chloramphenicol acetyl transferase activity observed in the resulting blastula stage sea urchin embryos, pRSVCAT and pSV2CAT plasmids were transferred by sperm and expressed successfully (Arezzo, 1989). DNA uptake and gene transfer by mouse epididymal spermatozoa was also reported by Lavitrano *et al.* in 1989 (Lavitrano *et al.*, 1989a). However, experiments carried out immediately after the publication of these findings were not able to replicate these results (Brinster, 1989). Additionally, Lavitrano *et al.* (1989b) later reported that they could not reproduce their previous results.

A subsequent experiment carried out by Gavora *et al.* (1991) found that plasmid DNA was taken up by mouse and chicken sperm, but no evidence of gene transfer into the embryos of either species was detected by DNA hybridisation analyses. This further added to the doubts of the efficacy of this method, and it appeared that the technique was unreliable. However, since these studies, there have been several investigations into the

uptake of DNA by sperm and some groups have reported success using sperm to transfer genes into the embryos of several species including insects, birds, mammals and amphibians (Gandolfi *et al.*, 1989; Hochi *et al.*, 1990; Perez, *et al.*, 1991; Lauria and Gandolfi, 1993; Habrova *et al.*, 1996; Sperandio, *et al.*, 1996).

As previously described in the Chapter 1 there is evidence to suggest that following incubation with sperm, DNA molecules are internalised by sperm cells. Such DNA becomes strongly associated with the sperm, resisting multiple washes, (Castro *et al.*, 1990; Horan *et al.*, 1991), digestion with DNase (Castro *et al.*, 1990; Atkinson *et al.*, 1991), gradient centrifugations, and prolonged dialysis (Horan *et al.*, 1991). Studies employing *in situ* hybridisation and ultrastructural autoradiography indicated that the DNA associated with sperm was in the region corresponding to the location of the nucleus (Brackett *et al.*, 1971; Horan *et al.*, 1991; Atkinson *et al.*, 1991; Camaioni *et al.*, 1992; Lavitrano *et al.*, 1992; Gandolfi, *et al.*, 1996) and that a portion of the DNA was internalised into the sperm nuclei (Francolini *et al.*, 1993).

While sperm transfer has the potential to be developed as an alternative gene transfer technology, simple incubation of sperm with DNA may not be capable of producing stable germ-line transgenic animals. Other manipulations of the DNA or sperm may be necessary to enhance the uptake and/or transfer of the DNA, or to increase the likelihood of integration of the DNA. One alternative method that has been used is sperm electroporation. Electroporation is the transient permeabilisation of the plasma membrane using a high voltage electric field. This reversible permeability is thought to occur because, as the cell membrane acts as an electrical capacitor through which current is unable to pass, local areas of breakdown occur followed by the formation of hydrophilic pores which then reseal (Weaver, 1993). DNA and other macromolecules have been transferred into a variety of cell types using this electrically induced membrane permeabilisation (Potter *et al.*, 1984; Andreason and Evans, 1988; Shigekawa and Dower, 1988; Förster and Neumann, 1989). Thus increasing the permeability of sperm (in the presence of DNA) using electroporation may increase the sperm uptake of DNA from solution, therefore increasing the amount of DNA taken up by the sperm and the

likelihood of gene transfer into embryos upon fertilisation.

Gagnè *et al.* (1991) studied the effects of electroporation on bovine sperm DNA uptake and found that electroporation enhanced foreign DNA uptake by sperm cells. In addition, the exogenous DNA was detected in 5 day old bovine embryos when they were fertilised by sperm electroporated with the foreign DNA (Gagnè *et al.*, 1991). Electroporation also increased the amount of DNA taken up by pig sperm; after multiple washes 1.6 times as much DNA remained following electroporation compared to incubation of the sperm with DNA only (Horan *et al.*, 1992). When the transfer of genes into chickens by sperm mediated methods was evaluated by Nakanishi and Iritani (1993), the localisation of DNA taken up by sperm was found to be effected by electroporation. More electroporated sperm were found to contain DNA in the acrosomal and posterior head region than non-electroporated sperm. However, when the sperm were used for fertilisations the efficiency of gene transfer of electroporated sperm (7/31) was lower than that of sperm only incubated with the DNA (14/30). Electroporation has also been shown to increase the amount of DNA taken up by abalone sperm (Sin *et al.*, 1995).

As the microinjection of fish eggs has met with many difficulties (Maclean *et al.*, 1987b), the use of sperm as a gene transfer vector is an attractive alternative for the production of transgenic fish. Unlike mammals, fish sperm are immobile in seminal plasma and fertilisation is external, making the manipulations involved in sperm gene transfer easier than in other animals. The potential of this technique has been tested in several fish species. Castro *et al.* (1990) reported that when mixed with carp sperm, plasmid DNA was taken up within 30 seconds, compared to the 15 to 30 minutes required for uptake by mammalian and avian sperm. Khoo *et al.* (1992) found zebrafish sperm to be capable of taking up plasmid DNA and transferring this into zebrafish embryos.

Gene transfer by electroporated sperm of common carp, African catfish and tilapia was reported by Müller *et al.* (1992). When non-electroporated sperm of these species were used no transfer was detected (Müller *et al.*, 1992). Tsai *et al.* (1995) demonstrated that electroporated (Baekonized) loach sperm take up DNA and used this technique to transfer a salmon growth hormone gene construct into loach.

A study by Patil and Khoo (1996) indicated that the amount of DNA taken up by zebrafish sperm was increased by electroporating the sperm. Additionally ultrastructural *in situ* hybridisation followed by autoradiography indicated that a portion of the DNA taken up by the sperm was internalised into the sperm nuclei.

Studies in our laboratory have shown that electroporated sperm can transfer circular pRSV-lacZ DNA into chinook salmon embryos, and that the frequency of transfer is dependent on field strength and pulse length (Sin *et al.*, 1993). Similar to the results of Müller *et al.* (1992), no transfer by non-electroporated sperm was observed. Sperm were exposed to field strengths of 250, 625 and 1150 V/cm in combination with single pulses of 7.7 and 18.6 ms. There appeared to be a threshold effect as below 625 V/cm and a pulse length of 18.6 ms, no transfer of pRSV-lacZ DNA was detected. Increasing the voltage over 625 V/cm did not increase the gene transfer efficiency.

Previous studies examining DNA uptake by electroporated sperm have not addressed in detail many of the factors affecting DNA uptake, and have only given limited indications of the optimal conditions for the uptake of DNA. Gagnè *et al.* (1991) electroporated cattle sperm with different field strengths (500, 1000, and 1500 V/cm) and capacitances (1 and 25 μ F), but did not find any difference in the retention of plasmid DNA between the different treatments. Nakanishi and Iritani (1993) obtained evidence, after electroporating chicken sperm over a range of field strengths (3,000 to 10,000 V/cm), that two pulses of 8000 V/cm resulted in the highest level of DNA uptake. The electroporation equipment used in these two studies differed; an exponentially decaying pulse was applied to cattle sperm (Gagnè *et al.*, 1991), while the chicken sperm were treated with an oscillating electric pulse with a sharp end spike (Nakanishi and Iritani, 1993). Due to the differences between the electric pulse characteristics, and possible species specific responses to electroporation, only limited comparisons can be made between these studies. Therefore, for every species it will be necessary to determine the optimal conditions for DNA uptake by electroporated sperm and will be dependent on the type of electroporation apparatus used.

There are a number of parameters that have been found to effect the efficiency of

cell transformation by electroporation. Increasing the strength of the electric field applied increases the efficiency of gene transfer into cells, up to the point where cell death outweighs any increase in the production of transfectants (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Increasing the concentration of the DNA, over a range of concentrations and up to several hundred micrograms per ml, has also been shown to increase the efficiency of transfection of electroporated cells (Chu *et al.*, 1987; Andreason and Evans, 1988; Anderson *et al.*, 1991). In a number of studies the application of multiple pulses has been shown to have a positive effect on gene transfer by electroporation (see Förster and Neumann, 1989, for a review). DNA uptake by chicken and abalone sperm has also been found to be promoted by two electric pulses (Nakanishi and Iritani, 1993; Sin *et al.*, 1995).

The data presented in this chapter cover an investigation into the uptake of circular and linear pRSV-lacZ plasmid DNA by electroporated and non-electroporated chinook salmon sperm. The aim was to test the effect of electroporation conditions on this uptake. The use of one or two electric pulses, with field strengths of 625 and 1000 V/cm was evaluated, as it had been shown that 625 V/cm represented a threshold below which gene transfer was unlikely (Sin *et al.*, 1993). The effect of the DNA concentration, (20 µg/ml or 100 µg/ml) mixed with the sperm was also assessed. To determine if any DNA was entering the sperm the effect of digesting the sperm with DNase after incubation with pRSV-lacZ DNA was examined. Since ultimately gene transfer methods aim for expression of the transferred DNA, it is important that the introduced DNA remain intact during the uptake process. Thus, three different DNA detection methods were employed as it was important not only to be able to detect low amounts of pRSV-lacZ DNA, but also to determine the condition of the DNA detected.

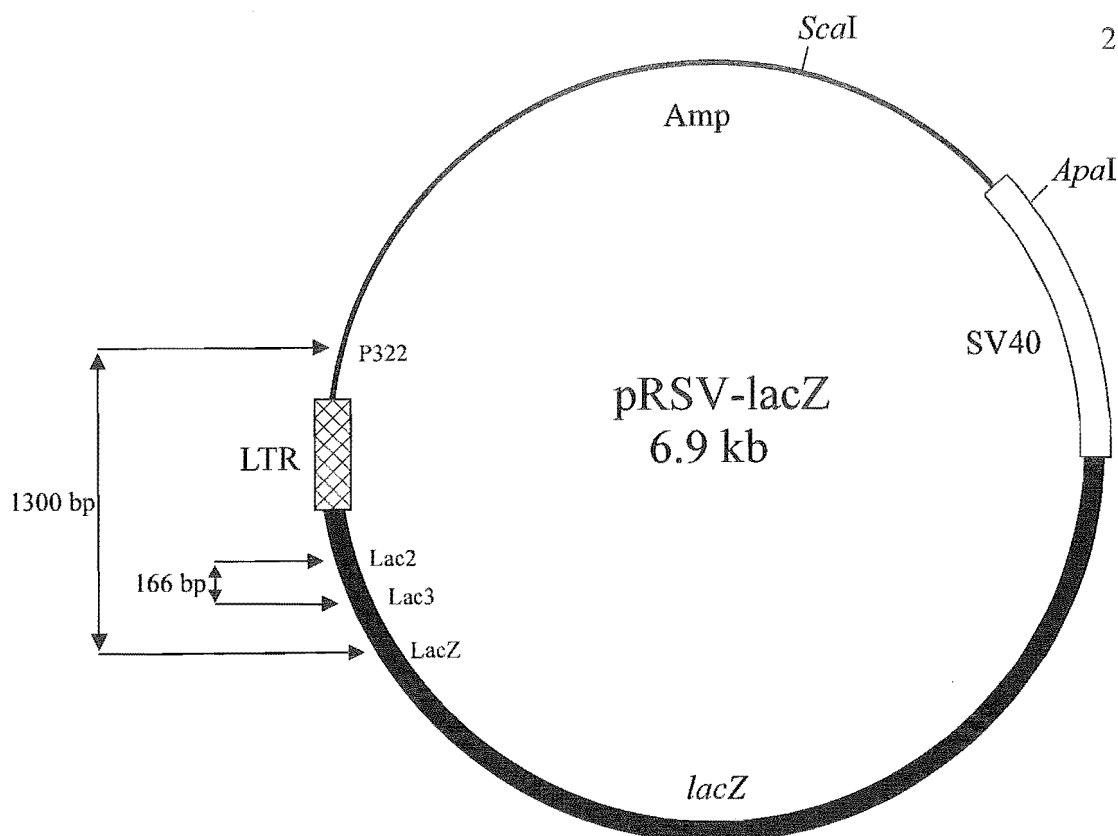


Figure 2.1. Schematic map of the plasmid pRSV-lacZ. The *lacZ* gene is shown as a solid thick line, the Rous Sarcoma Virus LTR as a shaded box, and the SV40 processing signal as an open box. The position of the ampicillin resistance coding region (Amp) is indicated. The primers P322 and LacZ were used in the PCR analysis to amplify a 1300 bp fragment in *fy* containing pRSV-lacZ DNA. The primers Lac2 and Lac3 were used to amplify an internal fragment (166 bp) of the *lacZ* gene for use as a probe. The restriction sites for *Apal*, and *Scal* are indicated. Not drawn to scale.

2.2 Materials and Methods.

2.2.1 Plasmid description.

The plasmid used in this study, pRSV-lacZ (Figure 2.1) (Altschmied, Schulz and Renkawitz, unpublished), consists of the ampicillin resistance gene and the origin of replication from the plasmid pBR322 joined to a hybrid eukaryotic expression unit. The expression unit is comprised of the Rous sarcoma virus long terminal repeat, the bacterial gene sequence coding for β -D-galactosidase (β -gal), and simian virus 40 (SV40) mRNA processing signals, including the small-t intron and early region polyadenylation site (Gorman *et al.*, 1983). To obtain linear plasmid DNA, pRSV-lacZ was cleaved at the single *Scal* site within the pBR322 sequence (Figure 2.1). The vector sequences were not removed from the pRSV-lacZ DNA as this plasmid has been shown to express adequately in this form after transfer into fish (Winkler *et al.*, 1991), and because large quantities of isolated insert would need to be purified for optimisation of the sperm electroporation procedure.

2.2.2 Isolation of plasmid DNA.

Plasmid DNA used for the sperm DNA uptake study was isolated using the following protocol. *Escherichia coli* (HB101) cells transformed with plasmid DNA were grown in 125 ml LB media (bactotryptone (10 g), bacto-yeast extract (5 g), sodium chloride (NaCl) (10 g), made up to 1 litre with water) containing 50 µg/ml ampicillin and incubated overnight at 37°C in a shaking incubator. The bacterial cells were recovered by centrifugation at 4500 rpm (Sorvall, GSA rotor) for 10 minutes at 4°C. The resulting pellet was resuspended in 5 ml of lysis solution (5 mg/ml lysozyme, 25 mM 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris), pH8, 10 mM ethylenediaminetetracetic acid (EDTA), 50 mM glucose) and incubated at room temperature for 10 minutes. Ten ml of alkaline lysis solution (0.2 N sodium hydroxide (NaOH), 1% sodium dodecyl sulphate(SDS)) was added and the suspension mixed until lysis was achieved. The released bacterial chromosomal DNA was precipitated by the addition of ice-cold high salt solution (3-5 M potassium acetate (KOAc), pH 5.2), and removed by centrifugation at 10,000 rpm (Sorvall, SS34 rotor) for 10 minutes at 4°C. The plasmid DNA was recovered from the supernatant by the addition of 0.6 volumes of isopropanol, incubated at room temperature for 1 hour, and centrifuged at 10,000 rpm (Sorvall, SS34 rotor) for 20 minutes. The resulting pellet was washed in cold 70% ethanol, dried, and resuspended in 2 ml of TE8 (10 mM Tris-hydrochloride (Tris-HCl), 1 mM EDTA, pH 8.0). Ribonuclease A (100 µg/ml) digestion was carried out for 1 hour at 37°C, followed by treatment with proteinase K at 100 µg/ml for 1 hours at 37°C. The solution was then extracted with an equal volume of TE8 buffered phenol and centrifuged at 7,000 rpm (Sorvall, SS34 rotor) for 5 minutes. The aqueous phase was extracted with chloroform/isoamyl alcohol (24:1) and the DNA precipitated by the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and two volumes of ethanol. After precipitation at room temperature for 1 hour, centrifugation (10,000 rpm, Sorvall, SS34 rotor) for 20 minutes, washing in cold 70% ethanol, and drying, the DNA was resuspended in TE8.

2.2.3 Electroporation of sperm.

Half a ml of milt (approximately 5×10^9 sperm) was diluted in a 1:1 ratio with $1 \times$ 2-hydroxyethyl piperazine-2-ethanesulfonic acid (HEPES) buffered saline (HBS) (pH 5.95, 21 mM HEPES, 137 mM NaCl, 50 mM potassium chloride (KCl), 0.7 mM disodium phosphate (Na_2HPO_4), 6 mM sucrose), containing circular or linear pRSV-lacZ DNA.

A Cell-Porator (System I) electroporation system (Bethesda Research Laboratories, (BRL)), which generates pulses with exponential decay, was used for the electroporation with a distance of 4 mm between the electrodes. The Cell-Porator electroporation apparatus consists of a pulse control unit, with an internal power supply, connected by a cable to the chamber safe (Figure 2.2). The electroporation chambers containing the cells to be shocked sit in ice in the chamber safe. The magnitude of the electric field (the field strength) and period that this is applied to the cells (the pulse length) are determined by the voltage and capacitance settings on the pulse control unit. The voltage setting is a continuous variable, and the Cell-Porator can be set to charge up to a maximum of 400 V. As the distance between the electrodes is 4 mm the strength of the resulting electric field (V/cm) is 2.5 times the voltage used. The length of the electric pulse is determined by the capacitance used and the resistance of the sample. For the buffer (HBS), temperature (0°C), and the volume (1 ml) used, the pulse lengths delivered by the Cell-Porator when the capacitance is set to 300, 800 and 1180 μF are 7.7, 18.6 and 27.4 ms respectively (according to the manufacturers instructions).

Following electroporation the ability of the sperm to be activated was assessed under a light microscope at $400 \times$ magnification. Five μl of sperm were placed onto a microscope slide to which a drop of water was added. If the sperm remained intact then the addition of water immediately initiated sperm motility (Billard and Cosson, 1992).

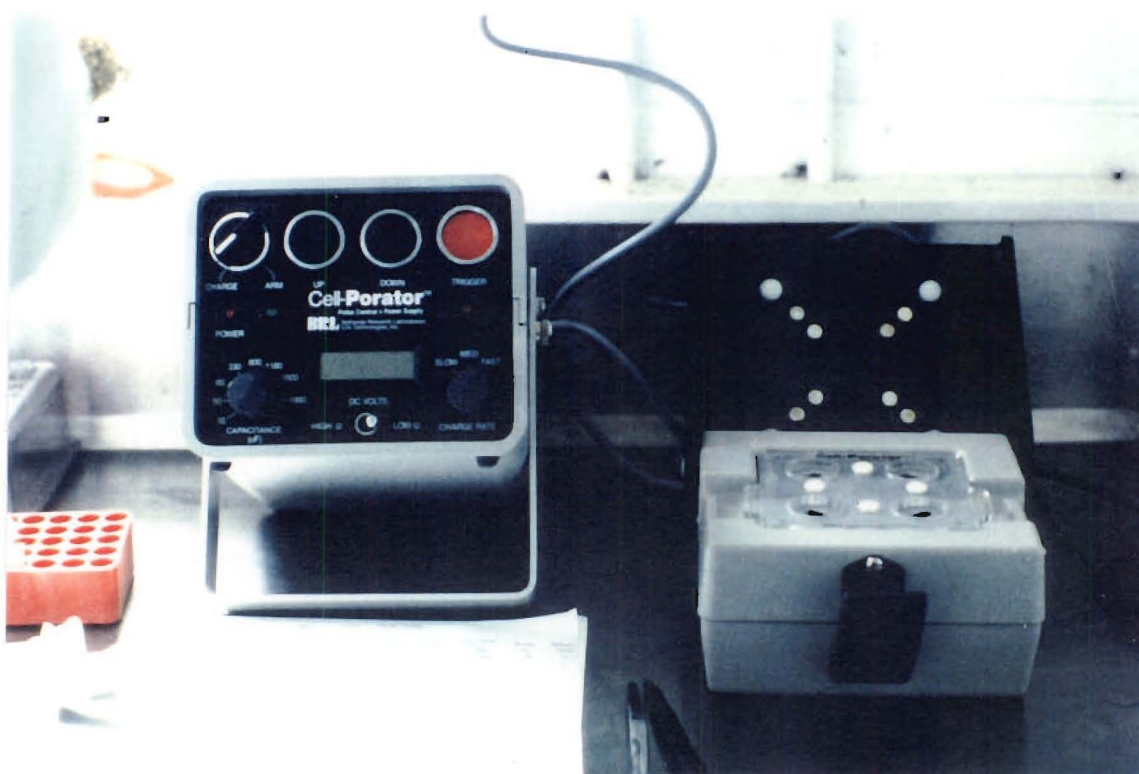


Figure 2.2. The Cell-Porator electroporation apparatus. The pulse control unit is pictured, with the chamber safe to the right.

The experimental approach used for the assessment of DNA uptake by electroporated salmon sperm is outlined in Figure 2.3. Two types of pRSV-lacZ DNA were used (circular or linear) at a concentration of either 20 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$. The effect of electroporation conditions was tested by increasing the field strength (625 V/cm or 1000 V/cm) and altering the number of pulses (1 or 2). A pulse length of 27.4 ms was used for the electroporation. After electroporation all samples were kept on ice for 10 minutes to allow DNA uptake and were then spun at 4,500 rpm (Jouan 14.11) for 10 minutes at 4°C. The supernatant (SN) was removed and kept for analysis. All sperm were resuspended in 1 ml of DNase digestion buffer (0.1 M sodium acetate pH 5.0, 5 mM magnesium sulphate) and half of each sample (500 μl) was digested with 50 units of DNase I for 2.5 hours at 37°C. The DNase was inactivated by the addition of 25 μl of 0.5 M EDTA and incubation at 65°C for 15 minutes. The digested and undigested samples

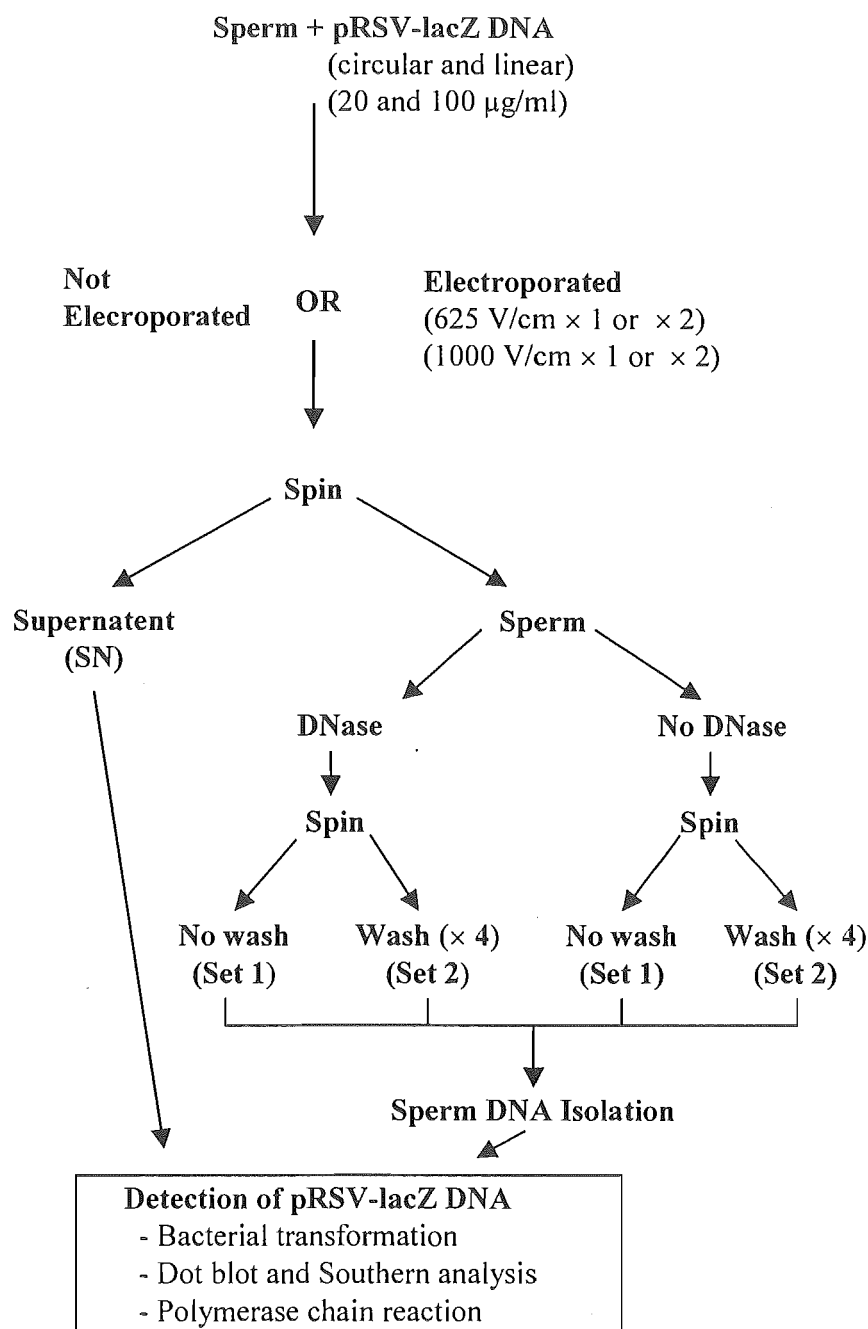


Figure 2.3. Outline of the method used to assess sperm DNA uptake. Two types of pRSV-lacZ DNA were used (circular or linear) at a concentration of either 20 µg/ml or 100 µg/ml. The effect of electroporation conditions was tested by increasing the field strength (625 V/cm or 1000 V/cm) and altering the number of pulses (1 or 2). A pulse length of 27.4 ms was used for the electroporation. After treatment all sperm were pelleted and the supernatant (SN) retained for analysis. All sperm were resuspended in 1ml of DNase digestion buffer and half of each sample (500 µl) was digested with DNase I. The digested and undigested samples were pelleted as before and resuspended in HBS. All sperm samples were once more divided into two aliquots; one set was stored at -20°C (set 1), while the other (set 2) was washed four more times in HBS buffer (washed samples) and then stored at -20°C. DNA was extracted from all sperm samples and analysed for the presence of pRSV-lacZ DNA.

were pelleted as before and resuspended in HBS. All sperm samples were once more divided into two aliquots; one set was stored at -20°C (set 1), while the other (set 2) was washed four more times in HBS (washed samples) and then stored at -20°C. DNA was extracted from all sperm samples and analysed for the presence of pRSV-lacZ DNA. Non-electroporated sperm were similarly treated with pRSV-lacZ DNA to assess DNA uptake.

The efficiency of the DNase I digestion was tested by incubating 100 µg, 50 µg, 25 µg, and 10 µg of circular pRSV-lacZ in 500 µl DNase digestion buffer with 50 units of DNase I at 37°C for 2.5 hours. The reaction was terminated by the addition of 25 µl 0.5 M EDTA and the samples heated at 65°C for 15 minutes. Samples without DNase, but containing equivalent DNA amounts, were also treated similarly. Plasmid DNA was recovered from the samples by ethanol precipitation and its presence assessed by agarose gel electrophoresis and bacterial transformation.

2.2.4 Isolation of DNA from sperm.

DNA was extracted from 50 µl of all sperm samples. The sperm aliquot was added to 500 µl proteinase K digestion buffer (10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1 mM EDTA, 0.5% v/v SDS), and proteinase K added to 300 µg/ml. This mixture was then incubated at 50°C for 16 hours, and extracted with an equal volume of TE8 buffered phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 8500 rpm (microfuge) for 5 minutes. The aqueous phase was extracted with chloroform/isoamyl alcohol (24:1) and the DNA precipitated by the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and two volumes of ethanol. After incubation overnight at room temperature the DNA was recovered by centrifugation at 12,000 rpm (microfuge) for 30 minutes. The DNA was then washed with ice cold 70% ethanol, dried and resuspended in 150 µl TE8.

2.2.5 Quantification of DNA.

DNA concentrations were determined by measuring the optical density at 260 nm (LKB Biochrom Ultraspectrophotometer II) or by comparing the intensity of fluorescence of samples after electrophoresis in agarose gels containing ethidium bromide (Sambrook *et al.*, 1989). Assuming that a reading of OD₂₆₀ equal to one corresponds to a DNA concentration of 50 µg/ml, the concentration of the samples was calculated. Alternatively as the fluorescence of DNA in the presence of ethidium bromide is proportional to the amount of DNA present, the concentration of samples was estimated relative to standards of known concentration after gel electrophoresis and staining in 5 µg/ml of ethidium bromide (Sambrook *et al.*, 1989).

2.2.6 Transformation of *E. coli* strain HB101.

Bacterial transformation was used to assess the association of circular pRSV-lacZ DNA with electroporated and non-electroporated sperm. The method was employed to detect any plasmid DNA molecules remaining in the supernatant samples or in DNA extracted from the sperm following electroporation with plasmid DNA. If plasmid DNA was present and intact, because it carries the ampicillin resistance gene, it could be detected after transformation by the appearance of a colony on an ampicillin agar plate.

One hundred µl of the supernatant samples (SN) and 100 µl of each sperm DNA sample, extracted from all samples incubated with circular pRSV-lacZ DNA, were used in the transformation assay. Two hundred µl of a competent *E. coli* (HB101) cell solution was mixed with the DNA solution and incubated on ice for 40 minutes. The cells were then heat-shocked at 42°C for 2 minutes and mixed with prewarmed L broth (bactotryptone (10 g), bacto-yeast extract (5 g), NaCl (5 g), made up to 1 litre with water) containing 50 µg/ml ampicillin. The mixture was incubated at 37°C for 60 minutes, 200 µl spread onto agar plates containing 50 µg/ml ampicillin and the plates incubated at 37°C overnight. The number of transformants obtained for each set of treatments was compared using the nonparametric Mann-Whitney test (Zar, 1984).

2.2.7 The Polymerase chain reaction (PCR).

The polymerase chain reaction (PCR) was used to detect plasmid DNA using the method of Saiki *et al.* (1988) with minor modifications. Two 20mer oligonucleotide primers, P322 and LacZ, were used to amplify a 1300 bp fragment of pRSV-lacZ (see Appendix 1 and Figure 2.1). Oligonucleotide primers were obtained from the Canterbury Health Laboratories (Christchurch). PCR was carried out in a 25 μ l volume containing 1 μ g of template DNA, 50 mM KCl, 10 mM Tris-HCl (pH 9), 0.1% Triton X-100, 0.2 mM each of the deoxyribonucleoside triphosphates (dNTPs); dTTP, dATP, dCTP and dGTP, one unit of *Taq* polymerase (Promega) and 0.8 mM each of two primers. Magnesium chloride was also included in the reaction buffer, usually at a concentration of 2 mM, this however, was dependant on the primer pair used (see Appendix 1). Hot start PCR was carried out by the addition of primers after the reaction components had been heated to 80°C. After an initial heating step of 94°C for 3 minutes, amplification was performed with a Grant Autogene II thermal cycling waterbath and consisted of 30 cycles of 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C. A final extension of 7 minutes was included. The PCR products were electrophoresed in 1.8% agarose gels in 0.5 \times TBE (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA) for 1 hour at 60V (40 mA) using a horizontal gel apparatus. The DNA was stained in 5 μ g/ml ethidium bromide and viewed under ultra-violet light (254 nm).

As a control, and to assess the ability of the extracted DNA to be amplified, a portion (311 bp) of the melanin concentrating hormone gene (MCH) of chinook salmon (Minth *et al.*, 1989) was amplified in a separate PCR reaction using the primers MCH1 and MCH2 (Appendix 1). The 311 bp MCH fragment was successfully amplified in all the sperm samples analysed (see Figure 2.4 for an example), showing that the sperm DNA could be successfully amplified and that the PCR reactions were reliable.

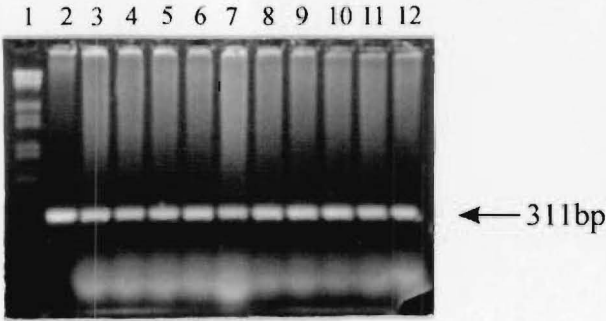


Figure 2.4. Example of the Melanin concentrating hormone gene PCR products amplified from sperm DNA samples. Lane 1: lambda DNA digested with *Eco* RI and *Hind* III (Fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 2: positive control, 311 bp MCH product amplified from salmon genomic DNA (0.2 μ g); 3 to 12 DNA extracted from salmon sperm used as the template.

2.2.8 Preparation of DNA for Southern analysis.

Both undigested high molecular weight and restriction endonuclease digested DNA was analysed by Southern blotting. DNA samples were digested by incubation with 5 units of restriction enzyme (Boehringer Mannheim) per μg of DNA in 30 μl of the appropriate digestion buffer (Boehringer Mannheim). Digestion was carried out using the conditions recommended by the manufacturer for 16 hours. DNA was electrophoresed for 20 hours at 25 volts (17 mA) in 0.8-1% agarose (BRL), containing 5 $\mu\text{g/ml}$ ethidium bromide, in $0.5 \times \text{TBE}$ using a horizontal gel apparatus. Lambda DNA (Boehringer) cleaved with *HindIII* (Boehringer), or *HindIII* and *EcoRI* (Boehringer), was used to provide size markers. DNA was visualised under ultra violet-light (254 nm).

2.2.9 Dot and Southern blotting and hybridisation.

For dot blot analysis sperm DNA in 10 μl of TE8 buffer was applied directly onto dry nitrocellulose filters (Schleicher and Schuell) (Aquadro *et al.*, 1986). DNA was transferred from agarose gels to nylon membrane using the method of Southern (1975) with modifications. Gels were pre-treated prior to transfer to depurinate and denature the DNA. Depurination was achieved by a 9 minutes wash in 0.2 M solution of hydrochloric acid. The gels were then rinsed in deionised water and denaturation carried out by one 15 and one 20 minute wash in denaturation/transfer solution (0.4 M NaOH, 1 M NaCl). Transfer to Zeta-probe nylon membrane (BioRad) was completed by capillary transfer in denaturation/transfer solution overnight. Membranes were washed in $2 \times \text{SSC}$ (300 mM NaCl, 30 mM tri sodium citrate), dried and then stored until hybridisation.

Membranes were prehybridised at 65°C in 1 mM EDTA, 0.5 M monosodium phosphate, pH7.2, 7% SDS and 0.1% (w/v) disodium pyrophosphate. Hybridisation to the probe was carried out at 65°C in the same solution for 16-20 hours. Following hybridisation, membranes were washed at room temperature, twice for 10 minutes in $2 \times \text{SSC}$, 0.1% disodium pyrophosphate, and twice for 10 minutes in $2 \times \text{SSC}$, 0.1% disodium pyrophosphate, 0.1% SDS. A final 45 minute wash at 65°C was carried out in $0.1 \times \text{SSC}$, 0.1% disodium pyrophosphate, 0.1% SDS. The membranes were exposed to X-ray film (Amersham Hyperfilm) with intensifying screens from 24 hours to 4 weeks at -80°C.

2.2.10 Preparation of probes.

Nick translation (Rigby *et al.*, 1977) was carried out at 16°C for 1 hour in a reaction mixture containing 0.25 µg of probe DNA, 20 nM each of the dNTPs; dTTP, dATP and dGTP, 5×10^{-3} ng of DNase I (Boehringer), 2.5 units of *E. coli* DNA polymerase I (Boehringer), 25 µCi of [α - 32 P] dCTPs (specific activity 3000 Ci/mmol) (Amersham) in a total volume of 12.5 µl of nick translation buffer (50 mM Tris, pH7.2, 10 mM magnesium sulphate, 1 mM dithiothreitol and 0.05 mg/ml bovine serum albumin). The reaction was stopped by the addition of EDTA to a concentration of 40 mM. The labelled DNA was then separated from the unincorporated nucleotides by chromatography in a bio-gel P60 sepharose column. Incorporation was checked by monitoring the column with a Geiger counter. Relative efficiencies of incorporation were assessed by reading the wash fraction in a Quick-Count 2000 bench top scintillation counter. Prior to hybridisation the probe DNA was denatured in a boiling water bath for 5 minutes followed by a 5 minute incubation on ice. The nick translated probes produced had a specific activity of $2 \times 10^7 - 10^8$ cpm/µg.

2.3 Results.

The data presented in this chapter have been published and the paper is included in Appendix 2 (Symonds *et al.*, 1994a).

2.3.1 Detection of DNA uptake by electroporated salmon sperm using bacterial transformation.

The HB101 transformation results are summarised in Table 2.1. The results indicated that plasmid DNA was associated with the sperm and that the association was enhanced by the electroporation of the sperm/DNA mixture. Prior to sperm DNA extraction the sperm were pelleted and the supernatant (SN) removed for transformation analysis (see Figure 2.3). Significant numbers of transformants (45 or 80) were only obtained in the supernatant from non-electroporated sperm samples, the number increasing when more pRSV-lacZ DNA (100 µg) was used in the electroporation medium. Only 2 out of the 14 supernatants analysed from the electroporated sperm samples produced a single transformant (Table 2.1).

Table 2.1. Total number of HB101 colonies detected after transformation with supernatant (SN) and sperm DNA.

Electroporation Conditions- Field Strength (V/cm)	Number of pulses	Amount of DNA (μ g)	DNase + or -	SN (N)	Sperm DNA Set 1 (N)	Sperm DNA Set 2 (N=4)
No Shock	0	20	-	45 (5)	0 (3)	0
No Shock	0	20	+		0 (3)	0
625	1	20	-	1 (3)	36 (3)	22
625	1	20	+		1 (3)	0
625	2	20	-	0 (3)	51 (3)	30
625	2	20	+		0 (3)	1
1000	1	20	-	0 (3)	32 (3)	37
1000	1	20	+		0 (3)	0
1000	2	20	-	0 (3)	43 (3)	42
1000	2	20	+		0 (3)	0
No Shock	0	100	-	80 (2)	6 (4)	NT
No Shock	0	100	+		0 (4)	NT
1000	2	100	-	1 (2)	120 (4)	286
1000	2	100	+		3 (4)	1

Set 1 = non-washed sperm samples. Set 2 = washed sperm samples. DNase (+ or -) indicates whether or not the sperm sample was treated with DNase prior to DNA extraction. The total number of transformants observed for all the replicates of a particular treatment is given. The number of replicates (N) is written in parentheses and refers to the number of independent sperm DNA samples analysed. In all cases a pulse length of 27.4 ms was used for the electroporation. NT = not tested.

The presence of pRSV-lacZ DNA in the sperm DNA samples was also indicated by the growth of bacterial transformants (Table 2.1). When non-electroporated sperm DNA samples were used, irrespective of the pRSV-lacZ DNA concentration used, only a small number of transformants was observed. In contrast all the electroporated (20 μ g/ml pRSV-lacZ) sperm DNA samples produced at least 10 HB101 colonies per replicate, and at the higher pRSV-lacZ concentration (100 μ g/ml) the number of transformants increased

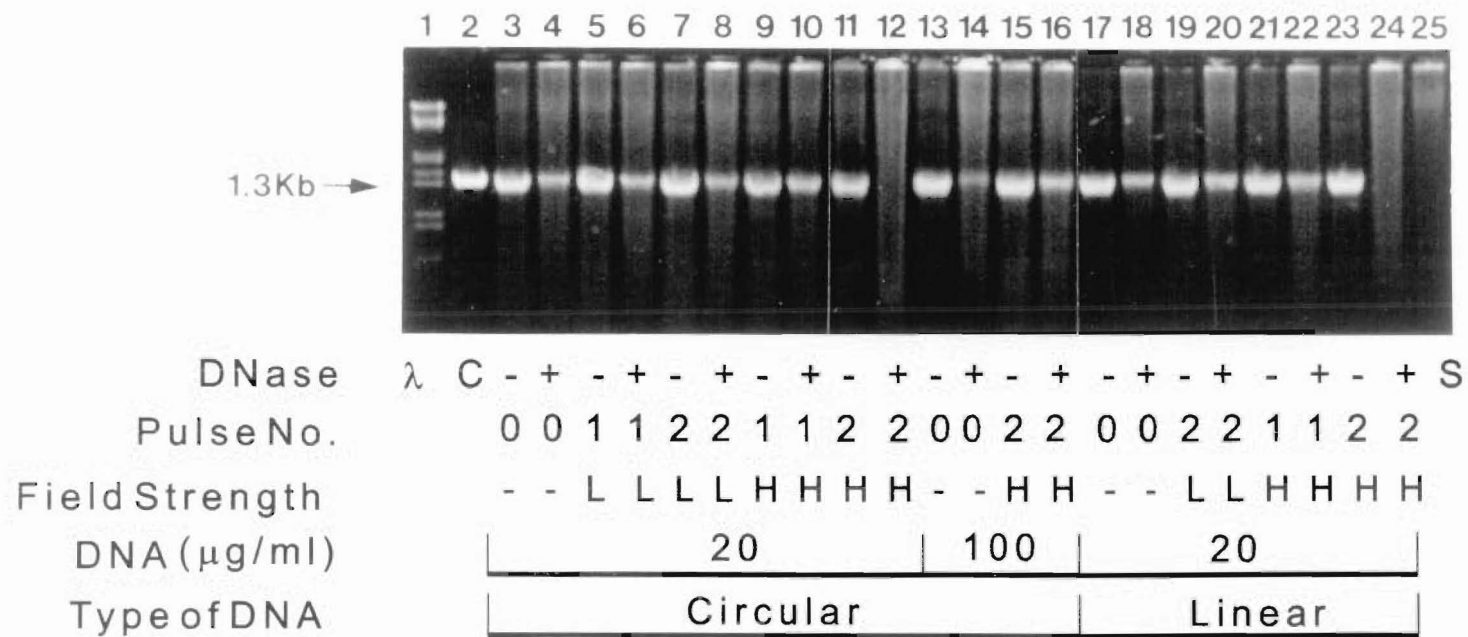
(Table 2.1). DNase digestion of the sperm prior to DNA extraction significantly decreased the number of transformants obtained. For most of the sperm samples electroporated with 20 µg of circular pRSV-lacZ no transformants were obtained following digestion (Table 2.1). Three out of 4 of the DNA samples extracted from sperm electroporated with 100 µg/ml pRSV-lacZ produced a single transformant after DNase digestion.

For the non-DNase digested sperm DNA samples the number of transformants obtained was independent of the field strength used. Doubling the number of pulses applied slightly increased the number of transformants obtained, but this difference was not significant ($p>0.2$). Additional washing of the sperm in HBS did not reduce the number of transformants significantly ($p>0.2$). Given that transformants were detected in the washed sperm samples (set 2), only these samples were analysed using the other detection methods.

2.3.2 Assessment of DNA uptake by electroporated salmon sperm using PCR.

PCR was used to screen for the presence of pRSV-lacZ DNA in the DNA extracted from all washed sperm samples (circular and linear pRSV-lacZ). A representative sample of these results is shown in Figure 2.5. The 1300 bp pRSV-lacZ PCR product was detected in all non-electroporated and electroporated DNA samples from non-DNase digested sperm, and also in most of the DNase digested samples (Figure 2.5). Two of the DNase digested treatments failed to produce a detectable 1300 bp PCR product: the 1000 V/cm 2 pulse samples electroporated with 20 µg/ml circular (lane 12) and 20 µg/ml linear pRSV-lacZ DNA (lane 24). In all other DNA samples extracted from DNase digested sperm the 1300 bp PCR product was observed, although the intensity of the amplified product was reduced in these samples compared to the non-DNase digested samples (Figure 2.5). Figure 2.5 shows only the results from one sperm DNA sample of each treatment, all other replicates (2 or 3) produced the same results.

Figure 2.5. PCR products detected in the DNA samples from sperm incubated with circular and linear pRSV-lacZ DNA using the primers P322 and LacZ. Lane 1: lambda DNA digested with *Eco*RI and *Hind*III (Fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 2: pRSV-lacZ DNA 1300 bp PCR product (positive control); 25: salmon sperm DNA only (negative control). The electroporation conditions (L = 625 V/cm, H = 1000 V/cm), the amount (20 µg or 100 µg/ml), and type of pRSV-lacZ DNA used (circular or linear), DNase digestion (- or +), and number of pulses (0, 1 or 2) are indicated for each lane.



2.3.3 Dot blot and Southern analysis.

Dot blot analysis was used to detect pRSV-lacZ DNA in all the washed sperm DNA samples (Figures 2.6a and 2.6b). 1 μg (- DNase, 20 $\mu\text{g/ml}$ samples), 0.5 μg (- DNase, 100 $\mu\text{g/ml}$ samples) or 10 μg (+ DNase samples) of sperm DNA were analysed in this way. The amount of DNA loaded was adjusted so that the resulting hybridisation intensities were at comparable levels. Almost no pRSV-lacZ DNA (less than 50 pg) was detected in the DNA samples from the non-electroporated sperm (Figure 2.6a). In the samples electroporated with circular pRSV-lacZ DNA (20 $\mu\text{g/ml}$) the amount of pRSV-lacZ DNA detected increased with increasing field strength and number of pulses, and more pRSV-lacZ DNA was detected when 100 μg of pRSV-lacZ DNA was used for the electroporation (dots 13 and 14, Figure 2.6a). DNase digestion of the electroporated sperm reduced the amount of pRSV-lacZ DNA detected approximately ten fold. The amount of pRSV-lacZ DNA remaining after DNase digestion of the sperm appeared to increase with increasing field strength and number of pulses. Dot blot analysis of the DNA extracted from sperm electroporated with linear pRSV-lacZ (20 $\mu\text{g/ml}$ only) showed similar results (Figure 2.6b). However, the effect of DNase digestion was more pronounced and the amount of DNA detected was reduced approximately twenty to fifty fold.

All the sperm DNA samples used for the dot blots were subsequently used for Southern analysis. 4 μg (- DNase samples) or 20 μg (+ DNase samples) of undigested sperm DNA, and 4-10 μg of the DNA from the electroporated sperm samples (- DNase only) digested with the restriction enzymes *HindIII* and *EcoRI* were analysed using this technique.

In Figure 2.7 (DNA from sperm incubated with circular pRSV-lacZ DNA) all non-electroporated samples produced only a faint signal following hybridisation to pRSV-lacZ DNA (lanes 5, 6, 16 and 17). DNA migrating with a similar mobility to the control pRSV-lacZ DNA was detected in all the non-DNase digested electroporated samples (lanes 8, 10, 12, 14 and 18) and overall, there appeared to be more pRSV-lacZ DNA present when the higher field strength was employed (lane 14) (lanes 4 to 11 were exposed to X-ray film 3.5 times longer than lanes 12 to 19).

Figure 2.6. Example of the dot blot analysis of DNA extracted from sperm (Set 2) incubated with (a) circular and (b) linear pRSV-lacZ DNA. 1 µg (- DNase, 20 µg/ml samples), 0.5 µg (- DNase, 100 µg/ml samples) or 10 µg (+ DNase samples) was applied to the filters.

(a) Dot number 15: 10 µg of negative control salmon genomic DNA. Dots 16 to 18: 200 pg, 100 pg and 50 pg of positive control pRSV-lacZ DNA. The other DNA samples were extracted from sperm treated with circular pRSV-lacZ DNA under the following conditions (the electroporation conditions are given as number of pulses × the field strength in V/cm):

<u>Dot number</u>	<u>DNA concentration (µg/ml)</u>	<u>Electroporation condition</u>	<u>DNase digestion (+ or -)</u>
1	20	No shock	+
2	20	No shock	-
3	20	1 × 625	+
4	20	1 × 625	-
5	20	2 × 625	+
6	20	2 × 625	-
7	20	1 × 1000	+
8	20	1 × 1000	-
9	20	2 × 1000	+
10	20	2 × 1000	-
11	100	No shock	+
12	100	No shock	-
13	100	2 × 1000	+
14	100	2 × 1000	-

(b) The samples were incubated with linear pRSV-lacZ DNA under the following conditions:

<u>Dot number</u>	<u>DNA concentration (µg/ml)</u>	<u>Electroporation condition</u>	<u>DNase digestion (+ or -)</u>
1	20	No shock	+
2	20	No shock	-
3	20	2 × 625	+
4	20	2 × 625	-
5	20	1 × 1000	+
6	20	1 × 1000	-
7	20	2 × 1000	+
8	20	2 × 1000	-

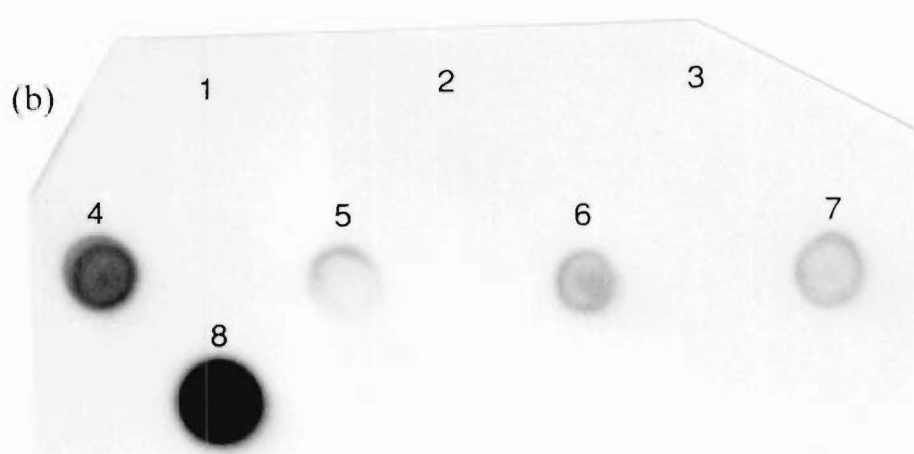
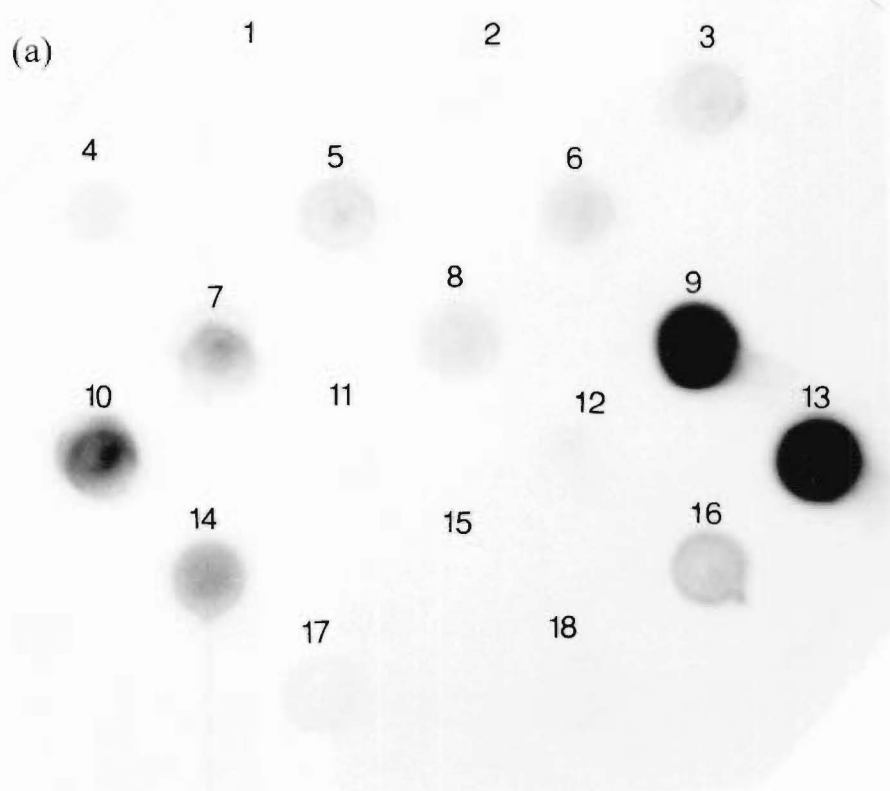
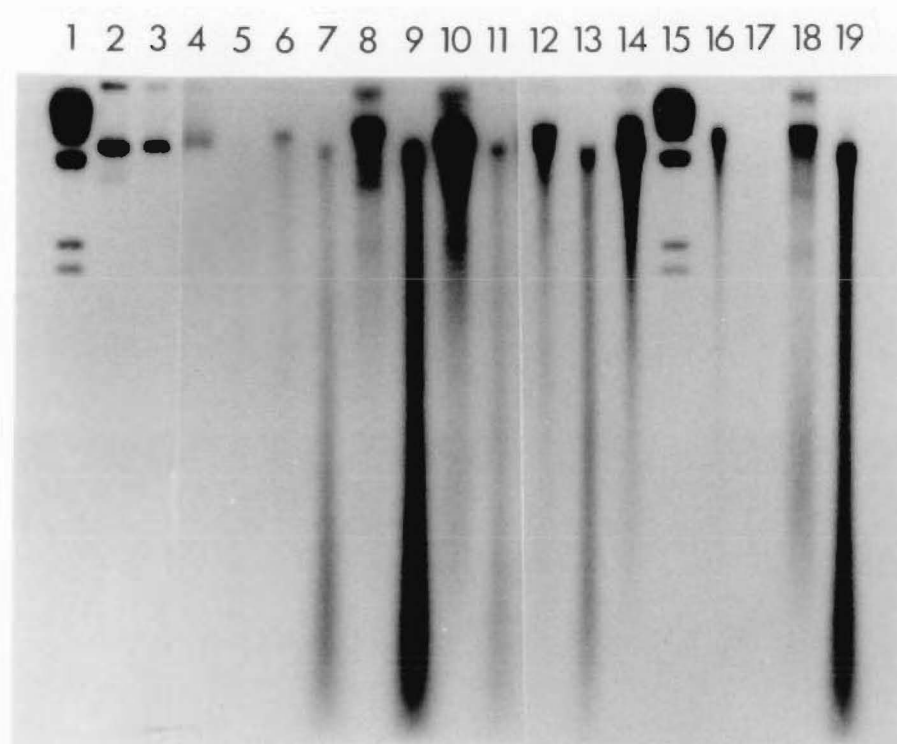


Figure 2.7. Southern analysis of undigested DNA extracted from sperm incubated with circular pRSV-lacZ DNA. Lanes 1 and 15: lambda DNA digested with *Hind*III (Fragment sizes (bp): 23130, 9416, 6557, 4361, 2322, 2027, 564, 125); 2: pRSV-lacZ DNA (1 ng); 3: pRSV-lacZ DNA (500 pg); 4: pRSV-lacZ DNA (100 pg). The electroporation conditions (L = 625 V/cm, H = 1000 V/cm), the amount of pRSV-lacZ DNA used (20 µg or 100 µg/ml), DNase digestion (- or +), and number of pulses (0, 1 or 2) are indicated for each lane. All samples are from the same membrane, but lanes 1-3 and 12-19 were exposed to X-ray film for 4 days, and lanes 4-11 for 14 days.



DNase	λ C C C + - + - + - + - + - λ - + - +																		
Pulse No.					0	0	1	1	2	2	1	1	2	2		0	0	2	2
Field Strength					-	-	L	L	L	L	H	H	H	H		-	-	H	H
DNA (µg/ml)					20											100			

All DNA samples extracted from DNase treated electroporated sperm (circular pRSV-lacZ DNA samples) produced a band of around 4-5kb as well as a smear below this (Figure 2.7, lanes 7, 9, 11, 13 and 19). The intensity of this band increased with increasing field strength and pulse number, and was the darkest in the sample electroporated with 100 μ g of pRSV-lacZ DNA (lane 19), even when the exposure time was shorter.

The DNA samples from sperm electroporated with linear pRSV-lacZ DNA (Figure 2.8) showed similar results. For the non-DNase treated sperm samples to which two pulses had been applied, two bands were observed (lanes 8 and 12) and more DNA was detected at the higher field strength (lane 12). For the single pulse sample (lane 10, 1000 V/cm) only one band was observed, and the hybridisation signal was much fainter. The dark bands visible in lanes 8, 10 and 12 had similar mobilities to pRSV-lacZ DNA (lanes 2 and 3). Following DNase digestion a single band was observed in the samples electroporated at 1000 V/cm (lanes 9 and 11). No DNA was detected in the 625 V/cm sample after DNase digestion (lane 7).

By comparing the relative intensities of the hybridisation signals on Southern blots of standard lanes and samples, the amount of DNA that was strongly associated with the electroporated salmon sperm was estimated. Following electroporation with 100 μ g/ml plasmid DNA, and following repeated washing, approximately 1.5 and 0.12 fg of DNA was associated with each sperm in the non-DNase and DNase digested samples respectively (Figure 2.7).

The non-DNase treated electroporated sperm DNA samples were digested with the restriction enzymes, *Hind*III and *Eco*RI (Figure 2.9). After *Hind*III digestion a single band (6.9kb) was expected for the samples electroporated with circular pRSV-lacZ, if no changes to the pRSV-lacZ plasmid had occurred (see Figure 2.1). In all samples a single band was observed, although the relative migration of this band varied between the different electroporation conditions (lanes 5 to 9). Three fragments were expected after *Eco*RI digestion (3.3kb, 2.4kb, and 1.2kb), and despite a slight difference in migration between the samples (lanes 13 to 17), the overall fragment pattern was similar to that obtained for the control pRSV-lacZ plasmid (lane 4).

Figure 2.8. Southern analysis of undigested DNA extracted from sperm incubated with 20 µg/ml linear pRSV-lacZ DNA. Lanes 1 and 13: lambda DNA digested with *Hind*III (Fragment sizes (bp): 23 130, 9416, 6557, 4361, 2322, 2027, 564, 125); 2: pRSV-lacZ DNA (1 ng); 3: pRSV-lacZ DNA (400 pg); 4: salmon sperm DNA only (20 µg) (negative control). The electroporation conditions (L = 625 V/cm, H = 1000 V/cm), DNase digestion (- or +), and number of pulses (0, 1 or 2) are indicated for each lane. The membrane was exposed to X-ray film for 14 days. The arrow shows the position of the high molecular weight band present in lanes 8 and 12.

1 2 3 4 5 6 7 8 9 10 11 12 13

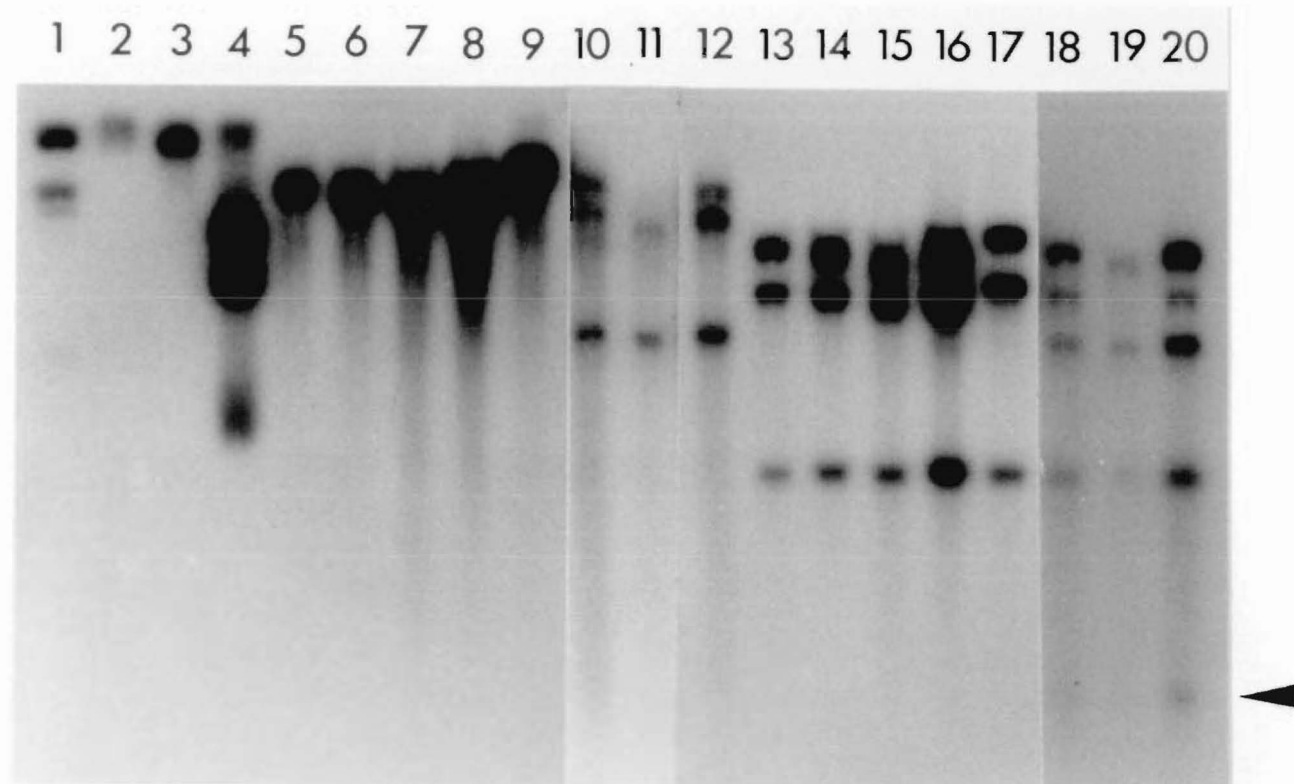


λ C C S

λ

DNase	+	-	+	-	+	-	+	-
Pulse No.	0	0	2	2	1	1	2	2
Field Strength	-	-	L	L	H	H	H	H

Figure 2.9. Southern analysis of sperm DNA digested with *EcoRI* and *HindIII*. Lane 1: lambda DNA digested with *EcoRI* and *HindIII* (Fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 2: undigested pRSV-lacZ DNA (1 ng); 3: *HindIII* digested pRSV-lacZ DNA (1ng); 4: *EcoRI* digested pRSV-lacZ DNA (5 ng). Lanes 5 to 12 contain sperm DNA digested with *HindIII*. Lanes 13 to 20 contain sperm DNA digested with *EcoRI*. The electroporation conditions (L = 625 V/cm, H = 1000 V/cm), type of pRSV-lacZ DNA used (circular or linear), and number of pulses (1 or 2) are indicated for each lane. Lanes 9 and 17 contain DNA extracted from sperm electroporated with 100 µg/ml pRSV-lacZ DNA, all other lanes contain DNA from sperm electroporated with 20 µg/ml. The arrow shows the position of the 0.5kb band observed in lanes 18, 19, and 20. All samples are from the same membrane, but lanes 1-9 and 12-17 were exposed to X-ray film for 12 hours, and lanes 10-11 and 18-20 for 24 hours.



	λ	C	H	E																
Pulse No.					1	2	1	2	2	2	1	2	1	2	1	2	2	2	1	2
Field Strength					L	L	H	H	H	L	H	H	L	L	H	H	H	L	H	H
DNA Type					<u>Circular</u>					<u>Linear</u>			<u>Circular</u>					<u>Linear</u>		
Enzyme					<u>HindIII</u>					<u>EcoRI</u>										

After *Hind*III digestion of DNA from samples electroporated with linear pRSV-lacZ two fragments were expected (4.9kb and 2.0kb) (Figure 2.9, lanes 10 to 12). The sample treated with a single pulse of 1000 V/cm produced 2 bands (lane 11), whereas the two sperm samples treated with double pulses (lanes 10 and 12) produced an additional band. Four fragments were expected following *Eco*RI digestion (3.3kb, 1.9kb, 1.2kb, and 0.5kb). In the single pulse sample (lane 19) four bands were observed, whereas in the double pulse samples (lanes 18 and 20) five bands were detected.

2.4 Discussion.

Overall, the results presented in this chapter show that electroporation enhances salmon sperm/DNA association. The HB101 transformation, dot blot, and Southern analyses detected only low levels of pRSV-lacZ DNA (circular or linear) in the DNA samples extracted from the non-electroporated sperm. The presence of pRSV-lacZ DNA in the supernatant from these samples implied that the DNA was easily removed from the non-electroporated sperm. In the electroporated sperm DNA samples pRSV-lacZ DNA was detected by all four detection methods used, and the binding of this DNA to the sperm appeared to be strong as it resisted multiple washing steps. Although the PCR analysis revealed no difference between the non-electroporated and electroporated samples, this probably reflects the greater sensitivity of this technique and its ability to detect femtogram amounts of DNA.

DNase was added to the sperm samples to degrade any pRSV-lacZ DNA remaining in solution or only loosely attached to the surface of the sperm. Although the bacterial transformation indicated that most of the pRSV-lacZ DNA was removed after DNase digestion, the PCR, dot blots and Southern showed that some pRSV-lacZ DNA remained in the sperm DNA samples. Therefore since not all pRSV-lacZ DNA was removed by exhaustive DNase digestion, some of the pRSV-lacZ DNA may be entering the sperm following electroporation. Alternatively association of the pRSV-lacZ DNA with the sperm plasma membrane may be sufficient to protect the DNA from contact with DNase. Several reports have also documented a strong association between plasmid DNA and sperm after incubation which resisted multiple washings (Castro *et al.*, 1990; Horan *et*

et al., 1991; Lauria and Gandolfi, 1993; Sin *et al.*, 1995) and digestion with DNase (Brackett *et al.*, 1971; Castro *et al.*, 1990; Atkinson *et al.*, 1991; Lauria and Gandolfi, 1993; Sin *et al.*, 1995). Gagnè *et al.* (1991) examined DNA uptake by electroporated bovine sperm, and showed that the DNA remaining after DNase digestion was, at the least, partly embedded in the sperm plasma membrane, if not completely inside it.

It was estimated that, following washing and DNase digestion, the amount of DNA associated with each sperm cell, was on average, 0.12 fg (16 copies). The actual amount of DNA available to be transferred into an embryo may be up to 1.5 fg (200 copies), if DNase sensitive DNA attached to the outside of the sperm cells is also transferred, in addition to the DNA presumably internalised. This figure may be higher if the viable sperm take up more DNA than the non-viable or lysed sperm. By comparison 3000 and 1000 copies of plasmid DNA were estimated to become associated with cattle and loach sperm following electroporation (Gagnè *et al.*, 1991; Tsai *et al.*, 1995).

The dot blot and Southern analysis showed an increase in the amount of pRSV-lacZ DNA associated with the non-DNase and DNase treated sperm when a double pulse of 1000 V/cm was applied. In contrast no pRSV-lacZ DNA was detected by PCR in these DNase digested samples when electroporated with a double pulse of 1000 V/cm with 20 µg/ml pRSV-lacZ DNA, although a product was obtained when 100 µg/ml pRSV-lacZ DNA was used. This suggests that under these conditions a proportion of the pRSV-lacZ DNA present was altered, and at the low concentration the amount of unmodified DNA was too low to be amplified to detectable levels by PCR. The exact nature of the change to the pRSV-lacZ DNA is not known, and could be due to the loss of the primer binding sites or other alterations to the DNA under these conditions. Similarly, the number of HB101 transformants obtained did not increase when the field strength and pulse number was increased, although more pRSV-lacZ DNA was present. Bacterial transformation is the least sensitive of the methods employed and selects for a functional ampicillin gene. Therefore any pRSV-lacZ DNA carrying a rearrangement in this region would not be detected. By contrast the condition of the DNA is less important when DNA hybridisation techniques are used, as changes to relatively small lengths of sequence will not render the DNA molecules undetectable, and this could account for the different results obtained

with the various methods employed.

In transgenic studies the form of the DNA being introduced into the host is important. In this study similar results were obtained when sperm were electroporated with circular or linear pRSV-lacZ DNA, and for both DNA types the amount of pRSV-lacZ DNA associated with the sperm increased with increasing field strength and number of pulses. Following restriction enzyme digestion of the pRSV-lacZ DNA recovered from sperm electroporated with linear pRSV-lacZ DNA, extra fragments were observed in the sperm samples electroporated with a double pulse. However, these extra fragments can be explained if the linear plasmid had re-ligated at the *ScaI* site; no other rearrangements were observed.

While evidence from other studies have shown that DNA can become strongly associated with sperm merely by incubation in solution together (Brackett *et al.*, 1971; Arezzo, 1989; Gandolfi *et al.*, 1989; Lavitrano *et al.*, 1989a; Hochi *et al.*, 1990; Atkinson *et al.*, 1991; Horan *et al.*, 1991; Perez, *et al.*, 1991; Camaioni *et al.*, 1992; Khoo *et al.*, 1992; Lavitrano *et al.*, 1992; Francolini *et al.*, 1993), it appears from this study and others (Gagnè *et al.*, 1991; Müller *et al.*, 1992; Nakanishi and Iritani, 1993; Tsai *et al.*, 1995; Sin *et al.*, 1995; Patil and Khoo, 1996), that electroporation of the sperm enhances this association and therefore increases the likelihood of gene transfer when these sperm are used for fertilisation. For example, when Tsai *et al.* (1995) examined the uptake of plasmid DNA by loach sperm, results similar to those presented in this chapter were obtained. After digestion with DNase only a very small amount of plasmid DNA was detected by Southern analysis in non-electroporated sperm samples, and only following electroporation were significant amounts of plasmid DNA detected. When the sperm were then used to fertilise loach eggs, gene transfer was detected only when electroporated sperm were used (Tsai *et al.*, 1995). Work previously carried out in our laboratory also showed that gene transfer by salmon sperm is increased by electroporation (Sin *et al.*, 1993). The observed efficiency of transfer into fry was dependent on field strength and pulse length, and below 625 V/cm no gene transfer was observed (Sin *et al.*, 1993).

The results presented in this chapter also demonstrate that electroporation conditions are important factors in determining the efficiency of sperm DNA uptake and

need to be optimised if efficient gene transfer frequencies are to be reliably obtained. Increasing the field strength, number of pulses, and concentration of plasmid DNA all significantly increased the amount of plasmid DNA associated with the sperm. One reason for the increased uptake at the higher field strength may be an increase in the number of sites on each sperm that experience membrane breakdown and become porated. Alternatively, as eluded to in mammalian cell transfection experiments (Weaver, 1993), because there is variation in the population of cells, as the field strength increases more reach the threshold level at which membrane breakdown occurs, and the percentage of permeable cells increases.

As in this study, Nakanishi and Iritani (1993) found that the magnitude of field strength used for electroporation of chicken sperm influenced DNA uptake and that more was taken up by the sperm if a second pulse was applied. A proposed explanation for increased transfer with increasing pulse number is a simple additive effect of the pulses: after the second pulse new sites of DNA/membrane contact are initiated and cause extra uptake (Förster and Neumann, 1989). Alternatively, the increase may be due to additional movement of DNA through the cell membrane at the same electroporated site initiated by the previous pulse (Förster and Neumann, 1989; Sukharev *et al.*, 1992).

In summary, if electroporated sperm are to be used to produce transgenic fish they must transfer the introduced DNA into the egg during fertilisation and the DNA must be capable of expressing in the developing fish. The data presented here indicate that the DNA is strongly bound to the electroporated sperm and may even be internalised as evidenced from other sperm uptake studies (Brackett *et al.*, 1971; Lavitrano *et al.*, 1992; Atkinson *et al.*, 1991; Camaioni *et al.*, 1992; Francolini *et al.*, 1993; Patil and Khoo, 1996). In addition expression of the DNA after electroporation was demonstrated as HB101 transformants were detected. Therefore, given the ability of electroporated salmon sperm to take up DNA, further work was carried out to assess the ability of electroporated sperm to transfer DNA into embryos and to optimise the electroporation conditions for sperm gene transfer. The results are presented in Chapter 3.

Chapter Three.

The effects of electrical and molecular variables on gene transfer by electroporated salmon sperm.

3.1 Introduction.

Before sperm electroporation can be used routinely for the production of transgenic salmon several parameters need to be optimised, such as the field strength, the length of the electric pulse, and the concentration of the introduced DNA. Therefore the optimal combination of these parameters needs to be experimentally determined in order to maximise the production of transgenic salmon.

In the use of electroporation for the transformation of mammalian cells in culture, the resulting number of transfected cells can be viewed as a trade off between cell mortality and transfer efficiency. As the strength of the electric field applied or the length of the pulse increases, mortality and transfer efficiency increase to a point where cell death outweighs any increase in the production of transfectants (Chu *et al.*, 1987; Shigekawa and Dower, 1988). The most successful electroporation of mammalian cells has been carried out using either high field strengths and short pulse lengths or low field strengths and relatively long pulse lengths (Chu *et al.*, 1987; Potter, 1989). Field strengths in the range of 500 to 1000 V/cm, with a combination of pulse lengths of 2 to 20 ms, are popular because of the ease of charging equipment to these relatively low voltages (Förster and Neumann, 1989).

Multiple pulses have been shown to increase the efficiency of gene transfer by electroporation (see Förster and Neumann, 1989, for a review). Up to five pulses at time intervals of several seconds have been successfully used (Förster and Neumann, 1989). Sukharev *et al.* (1992) found that DNA transfer into Cos-1 cells was greater when a second pulse with an amplitude lower and pulse length longer than that of the initial pulse was used. The additive effect was similar irrespective of whether the time between the 2 pulses was 0.1 ms or 1 s. When sperm mediated methods were assessed by Nakanishi and Iritani (1993), the application of a two electric pulses was also found to promote DNA

uptake by chicken sperm. Similarly, the data presented in Chapter 2 showed that increasing the pulse number increased the amount of DNA taken up by electroporated salmon sperm.

Electroporation media with high conductivity, such as HBS, are commonly used for electrotransfection of mammalian cell lines (Andreason and Evans, 1988; Förster and Neumann, 1989). The resulting field strength during electroporation is inversely related to the conductivity of the medium (Andreason and Evans, 1988). By altering the ionic strength of the buffer it is possible to expose cells to higher field strengths when the upper level of the charging equipment has been reached. Such changes to the physical parameters will change the optimal voltage for transfection (Andreason and Evans, 1988).

Experiments on gene transfer into cultured cells have demonstrated that the efficiency of stable transformation is on the whole greater with linear DNA than supercoiled circular plasmid DNA (Potter *et al.*, 1984; Andreason and Evans, 1988; Förster and Neumann, 1989). The reason for this difference is unclear; it may result from the more efficient introduction of the linear form into the cell, or from the greater probability of linear DNA integrating into the genomic DNA. It is hypothesised that the more rod like linear molecules pass through smaller pores in the cellular membrane more readily than the globular structures of supercoiled DNA molecules (Förster and Neumann, 1989). Alternatively this difference in efficiency may reflect a decreased efficiency for supercoiled DNA to reach the nucleus (Andreason and Evans, 1988). In the production of transgenic mice linear DNA has also demonstrated greater transfer efficiencies and integration rates over circular DNA (Brinster *et al.*, 1985).

Most transgenic fish studies have not compared the efficacy of using different DNA forms. Some investigators have found the production of transgenic fish to be more efficient by microinjecting linear over circular DNA into fish eggs (Chourrout *et al.*, 1986; Penman *et al.*, 1990). However, successful transfer of supercoiled plasmid DNA has also been reported (Rokkones *et al.*, 1989; Stuart *et al.*, 1990). Additionally, Chong and Vielkind (1989) did not find any difference in gene retention between linear and circular DNA microinjected into medaka eggs.

Given that it is unclear which topological form of DNA is more effective when used to produce transgenic fish, and more efficient transfer of circular DNA into cells using electroporation has been reported (for example, Sollazzo *et al.*, 1988), it was pertinent in this study to compare the relative efficiency of transferring linear and circular plasmid DNA into salmon by electroporated sperm.

The addition of carrier DNA, nonspecific high molecular weight DNA, has also been found to increase the efficiency of electrotransfection experiments (Chu *et al.*, 1987; Förster and Neumann, 1989; Spencer, 1991). For example, Chu *et al.* (1987) found that the inclusion of salmon sperm DNA, 1 to 6 kb in size, had a positive effect on the transfection of mammalian cells. This effect increased as the concentration of the carrier DNA was increased from 250 to 500 µg/ml, although further increase resulted in decreasing transfection efficiencies. It is thought that the excess carrier DNA protects the plasmid DNA from degradation by intracellular nucleases by a swamping effect (Förster and Neumann, 1989). Whereas this process may play some role in the observed increase, the presence of carrier DNA may also have a positive effect on the actual passage of DNA into the cells. This was implicated by the carrier DNA mediated increase in permeability observed by Sukharev *et al.* (1992). The uptake of fluorescent dextran by electroporated cells increased when calf thymus DNA was added, and was shown to be dependant on the concentration of the carrier DNA.

DNA uptake by electroporated cells is known to occur in the period following the exposure of cells to the electric pulse, and not during the relatively short period of time that the electric field is applied. Incubation of the cell/DNA mixture for approximately 10 minutes is necessary for uptake (Förster and Neumann, 1989). The temperature of the post-electroporation incubation is also important. Incubation at 0°C prolongs the period when the pores are open (Förster and Neumann, 1989; Spencer, 1991). The permeability of the membrane can be maintained in this way from several minutes to 1 hour (Förster and Neumann, 1989). Therefore, in an attempt to take advantage of a prolonged permeable state of electroporated cells, the time that the salmon sperm cells were incubated on ice after the electric shock was extended in this study to determine if this would increase the

uptake and gene transfer efficiency.

The efficiency of transfection by electroporation has been shown to increase with increasing DNA concentration (Chu *et al.*, 1987; Andreason and Evans, 1988; Anderson *et al.*, 1991). Uptake of DNA by electroporated salmon sperm was also shown to be more efficient when the concentration of the DNA incubated with the sperm was increased to 100 µg/ml (Chapter 2). Khoo *et al.* (1992) obtained successful transfer by zebrafish sperm incubated with 200 µg/ml of DNA, and gained evidence that concentrations above this may be detrimental to the sperm.

Work by Müller *et al.* (1992) indicated that electroporated sperm were capable of transferring DNA into common carp, African catfish and tilapia, with 2 to 4% of the developing fry containing the introduced DNA. While these results are encouraging the transfer efficiencies are relatively low. The motility of the electroporated sperm samples was maintained at the relatively high level of 70-80% compared to non-electroporated controls, suggesting that optimal parameters had not been reached with the exponential decay pulses of 2500 V/cm \times 7 ms used (Müller *et al.*, 1992).

The results in Chapter 2 and Sin *et al.* (1993), indicated that electroporated salmon sperm can take up DNA and pass this on to the developing embryo. Therefore the next step was to assess the repeatability of the technique, to try to improve transfer efficiencies and increase the likelihood of gaining actual germ line transgenic salmon using this technique. The main aim of the work presented in this chapter was to test the effect of the following variables on gene transfer efficiency: the addition of carrier DNA; the strength of the electric field (625 V/cm and 1000 V/cm); the length of time this field was applied to the sperm (pulse length, 18.6 and 27.4 ms); the number of pulses (0, 1 and 2); the type of DNA incubated with the sperm (ie. different plasmids, pRSV-lacZ and pSV2CAT); and the form of the DNA (circular and linear). Field strengths of over 625 V/cm and pulse lengths above 7.7 ms were used to electroporate the sperm as it had been shown that this represented a threshold below which gene transfer by electroporated salmon sperm was unlikely (Sin *et al.*, 1993). Uptake of DNA by electroporated salmon sperm using these conditions had also been demonstrated (Chapter 2). The effect of increasing the DNA

concentration from 20 µg/ml to 100 and 200 µg/ml was also determined. As higher field strengths, longer pulse lengths and multiple pulses are likely to lead to more cell lysis and decreased sperm viability the motility of the sperm was monitored.

3.2 Materials and Methods.

3.2.1 Plasmids.

Two plasmids were used in this study, pRSV-lacZ (see section 2.2.1 for a description of pRSV-lacZ) and pSV2CAT (Figure 3.1). Like pRSV-lacZ, pSV2CAT consists of the ampicillin resistance gene and the origin of replication from plasmid pBR322 joined to a hybrid eukaryotic expression unit. In pSV2CAT, the expression unit is composed of the SV40 early region promoter, the chloramphenicol acetyltransferase (CAT) gene, and the SV40 mRNA processing signals (Gorman *et al.*, 1982). The plasmid used was the pSV2CAT^R form, as opposed to pSV2CAT^S, as it has an extra 182 bp containing the CAT core promoter region (Gorman *et al.*, 1982). It will be referred to as pSV2CAT from now on. In 1992 linear plasmid DNA was obtained by restriction digestion of pRSV-lacZ with *ScaI* (Figure 2.1). In 1993 linear plasmid DNA was obtained by restriction digestion of pRSV-lacZ and pSV2CAT with *ApaI* (Figures 2.1 and 3.1).

3.2.2 Isolation of plasmid DNA.

Plasmid DNA used during the 1992 breeding season was isolated using the method outlined in section 2.2.2. Plasmid DNA used during the 1993 breeding season was isolated using the MagicTM Maxipreps purification system (Promega). *Escherichia coli* (DH5a) cells transformed with plasmid DNA were grown in 500 ml LB media containing 50 µg/ml ampicillin and incubated overnight at 37°C in a shaking incubator. The bacterial cells were then recovered by centrifugation at 4500 rpm (Sorvall, GSA rotor) for 10 minutes at 4°C. The cell pellet was resuspended in 15 ml cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml ribonuclease A) and the bacterial cells lysed by the addition of 15 ml cell lysis solution (0.2M NaOH, 1% SDS) followed by mixing by inversion. The mixture was then neutralised by the addition of 15 ml of 2.55 M

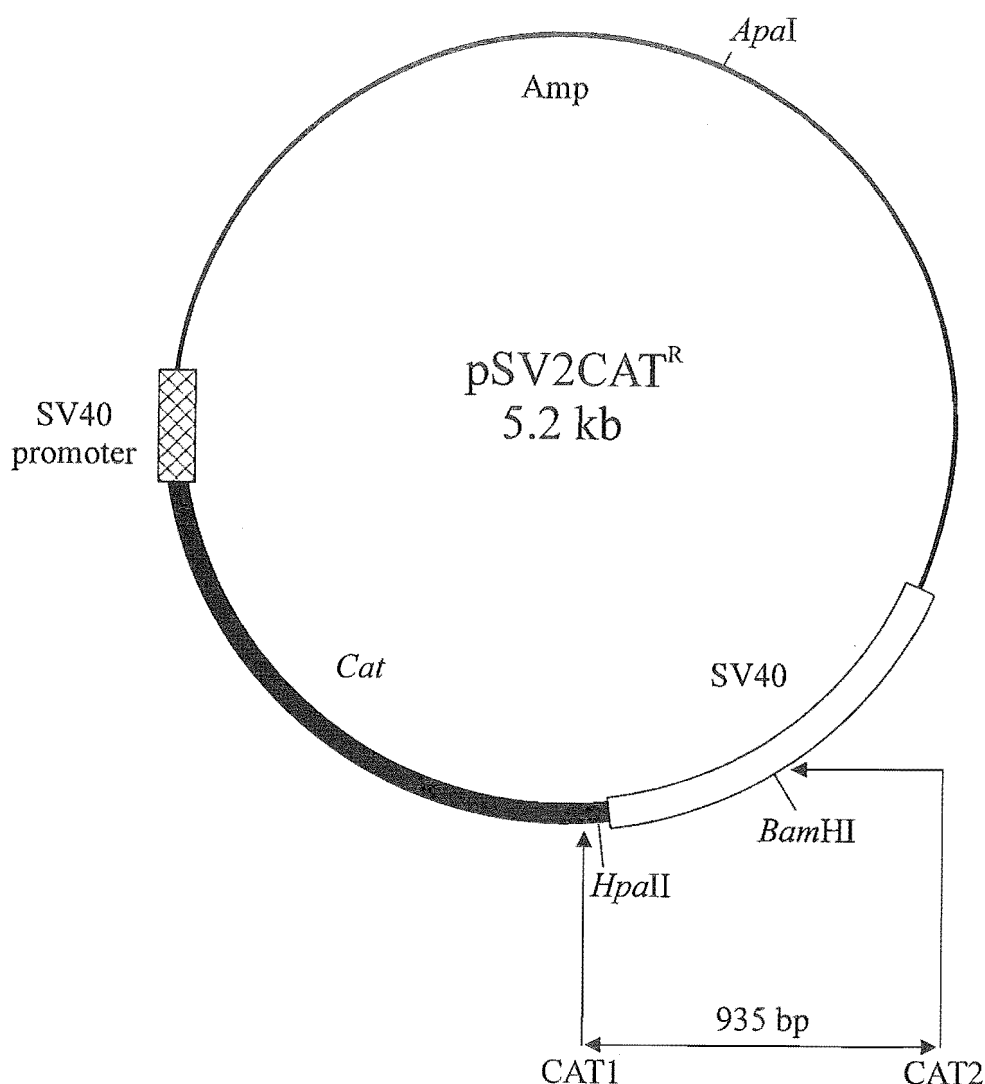


Figure 3.1. Schematic map of the plasmid pSV2CAT. The *Cat* gene is shown as a thick line, the SV40 early region promoter as a shaded box, and the SV40 processing region as an open box. The position of the ampicillin resistance gene (Amp) is indicated. The primers CAT1 and CAT2 were used in the PCR analysis to amplify a 935 bp fragment. Probe DNA used to detect the PCR product was prepared by removing the primer binding sites by digestion with *HpaII* and *BamHI*. The restriction site for *Apal* is indicated. Map not drawn to scale.

potassium acetate (pH 4.8). The released bacterial chromosomal DNA was pelleted by centrifugation at 11,000 rpm (Sorvall, SS34 rotor) for 15 minutes at 4°C. The plasmid DNA was recovered from the supernatant by the addition of 0.6 volumes of isopropanol followed by centrifugation at 11,000 rpm (Sorvall, SS34 rotor) for 15 minutes. The resulting pellet was resuspended in 10 ml of "Magic Maxipreps DNA purification resin" and this slurry loaded into a Magic Maxicolumn. The DNA/resin mix was washed by the addition of 13 ml of column wash solution (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 50% v/v ethanol) and the application of vacuum to the column. This wash

step was repeated with a further 12 ml of column wash solution. The DNA/resin mix was then washed in the same way with 5 ml 80% isopropanol and then dried by centrifugation for 5 minutes at 2500 rpm (Jouan GR412), in a swinging bucket rotor. To elute the DNA from the resin 1.5 ml of TE8, preheated to 65°C, was added to the column followed by a 1 minute incubation and centrifugation as before.

3.2.3 Electroporation of sperm.

Sperm were electroporated using a method similar to that outlined in section 2.2.3. Half a ml of milt (approximately 5×10^9 sperm) was diluted in a 1:1 ratio with $1 \times$ HBS containing circular or linear plasmid DNA. In the 1992 season the sperm were electroporated at different field strengths (625 V/cm and 1000 V/cm), pulse lengths (18.6 ms and 27.4 ms), and with a single, double, or triple pulse with 20 µg/ml of pRSV-lacZ or pSV2CAT DNA (Table 3.1). Based on the most successful conditions from 1992, in the 1993 season the sperm were electroporated with one pulse of 1000 V/cm \times 18.6ms or with two pulses of 1000 V/cm \times 27.4 ms with 20, 100 or 200 µg/ml of pRSV-lacZ or pSV2CAT DNA (Table 3.2).

After electroporation, sperm were incubated on ice for 10 minutes, and were then used to fertilise 300 freshly stripped eggs. The milt and eggs were collected from sea-run chinook salmon returning to the Silverstream hatchery, located on the Kaiapoi river approximately 20 km north-west of Christchurch, New Zealand. In both seasons the milt from one male was used for all the electroporations and fertilisations. Eggs from 3 females were used in 1992 and from 5 females in 1993.

3.2.4 Sperm motility.

In 1992 the motility of sperm samples was assessed with help from Dr. Iris Sin using a Makler Counter (Sefi Instruments). Samples were activated with salmon coelomic fluid and sperm motility was estimated within 15 seconds by counting the number of motile sperm and then the total number of sperm cells in a gridded area.

Table 3.1. Effect of electroporation conditions on gene transfer into fry- 1992 breeding season. Plasmid DNA was detected by PCR followed by Southern analysis of PCR products, in DNA extracted from 12 week old fry. Sperm were incubated with HEPES buffered saline (HBS) to a final concentration of 0.5 × or 1.0 ×, with circular (C) or linear (L) DNA of pRSV-lacZ (LacZ) or pSV2CAT (CAT). The plasmid DNA concentration was 20 µg/ml for all treatments. N = number of fry analysed.

Electroporation Conditions: Field Strength (V/cm) × Pulse length (ms)	Number of pulses	HBS: 1 × DNA: LacZ, C Positive Fry/N (%)	HBS: 0.5 × DNA: LacZ, C Positive Fry/N (%)	HBS: 0.5 × DNA: LacZ, L Positive Fry/N (%)	HBS: 0.5 × DNA: CAT, C Positive Fry/N (%)
No shock	0	-	0/18 (0%)	0/20 (0%)	3/20 (15%)
625 × 18.6	1	4/28 (14%)	3/18 (17%)	5/20 (25%)	-
625 × 27.4	1	6/22 (27%)	2/20 (10%)	0/20 (0%)	-
1000 × 18.6	1	-	12/31 (39%)	7/22 (32%)	-
1000 × 27.4	1	-	0/20 (0%)	1/21 (5%)	10/35 (28%)
625 × 27.4	2	-	-	11/44 (25%)	-
1000 × 27.4	2	-	-	22/50 (44%)	17/20 (85%)
625 × 27.4(2) + 18.6(1)	3	-	0/22 (0%)	-	-

Table 3.2. Effects of electroporation condition and DNA concentration on the efficiency of gene transfer into fry-1993 breeding season. Sperm were electroporated with circular (C) or linear (L) DNA of pRSV-lacZ (LacZ) or pSV2CAT (CAT). The electroporation conditions were no electroporation (0), 1 pulse 18.6 ms long of 1000 V/cm (1) and 2 pulses 27.4ms long of 1000 V/cm (2). Sample size for each treatment was 30 fry. NT = Not Tested

DNA concentration ($\mu\text{g/ml}$)	No. of pulses	Field strength (V/cm) \times Pulse length (ms)	Number of fry, out of 30, found to contain plasmid DNA (%)			
			LacZ,C	LacZ,L	CAT,C	CAT,L
100	0	-	6 (20)	0	3 (10)	NT
200	0	-	0	0	2 (6.7)	2 (6.7)
20	1	1000 \times 18.6	0	6 (20)	1 (3.3)	1 (3.3)
100	1	1000 \times 18.6	1 (3.3)	0	2 (6.7)	2 (6.7)
200	1	1000 \times 18.6	3 (10)	0	7 (23.3)	11 (36.7)
20	2	1000 \times 27.4	10 (33.3)	4 (13.3)	6 (20)	1 (3.3)
100	2	1000 \times 27.4	5 (16.7)	2 (6.7)	1 (3.3)	5 (16.7)
200	2	1000 \times 27.4	5 (16.7)	2 (6.7)	10 (33.3)	14 (46.7)

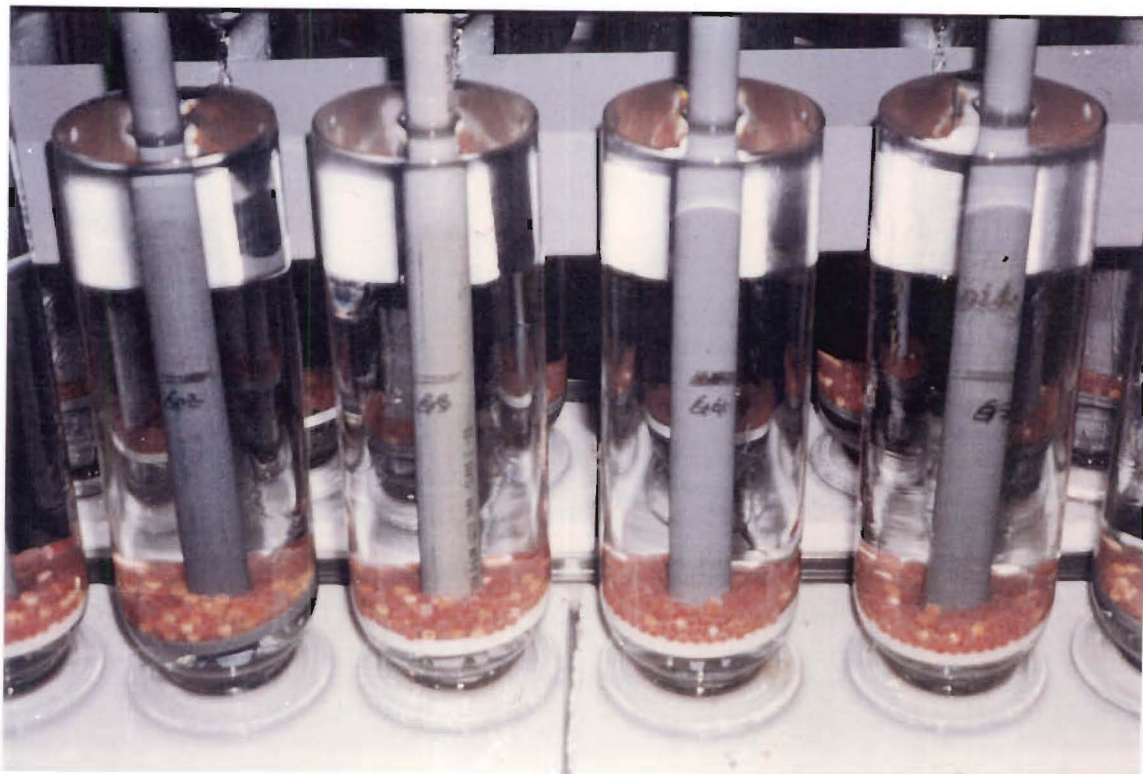


Figure 3.2 Incubation jars containing developing chinook salmon embryos.

3.2.5 Salmon rearing.

Salmon embryos were maintained in glass incubating jars supplied with $11 \pm 1^\circ\text{C}$ stream water (Figure 3.2). The embryos were cultured to the fry stage (12 weeks) before collection for analysis.

3.2.6 Isolation of fry DNA.

At twelve weeks post-fertilisation (1992 and 1993), at the stage of complete yolk sac absorption, fry were collected, snap frozen in liquid nitrogen and stored at -80°C . Approximately 1 fifth of each fry (the tail region) was cut off while still frozen, and partially ground in liquid nitrogen followed by homogenisation in 250 μl of proteinase K digestion buffer (10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1 mM EDTA, 0.5% v/v SDS). The volume was made up to 500 μl with proteinase K digestion buffer, proteinase K was added to 400 $\mu\text{g}/\text{ml}$ and mixed with the homogenate. This mixture was then incubated at 50°C for 16 hours, and extracted with an equal volume of TE8 buffered phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm (microfuge)

for 5 minutes. The aqueous phase was extracted with chloroform/isoamyl alcohol (24:1) and the DNA precipitated by the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and two volumes of ethanol. After incubation overnight at room temperature the DNA was recovered by centrifugation at 12,000 rpm (microfuge) for 30 minutes. The DNA was then washed with ice cold 70% ethanol, dried and resuspended in 70 μ l TE8.

3.2.7 Isolation of DNA from embryos.

In the 1993 spawning season the transfer of plasmid DNA into embryos was assessed. Embryo development was arrested at different stages by immersion in liquid nitrogen and embryos then stored at -80°C . Table 3.3 shows the ages of embryos collected and gives a description of each developmental stage. Genomic DNA was extracted using a modification of the method described by Chourrout *et al.* (1986). The embryos were thawed in ice cold salmo saline (111 mM NaCl, 2.7 mM KCl, 0.9 mM calcium chloride (CaCl_2), 0.4 mM magnesium chloride (MgCl_2), 2.4 mM sodium bicarbonate (NaHCO_3), 5.6 mM glucose, Burton *et al.*, 1975) and the chorion removed. To remove the yolk the embryos were washed twice in ice cold salmo saline. If individual embryos were too small to manipulate, this washing was accomplished by spinning at 3,400 rpm (Jouan 14.11) for 5 minutes at 4°C , and then removing the supernatant. The tissue was homogenised in approximately six volumes of lysis buffer (5 M guanidine isothiocyanate, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5, 8% v/v mercaptoethanol). For embryos 11 days old and younger, to assist the precipitation of low amounts of DNA, 1 microgram of salmon genomic DNA was added to the homogenates. 8 M lithium chloride was then added to give a final concentration of 3.44 M and the homogenate incubated at 4°C for at least 12 hours to precipitate the RNA. The RNA and cellular debris were removed by centrifugation at 12,000 rpm (microfuge) for 30 minutes. The DNA was then precipitated from the supernatant by the addition of two volumes of ethanol, followed by incubation at room temperature for 16 hours. The DNA was recovered by centrifugation at 12,000 rpm (microfuge) for 30 minutes, washed with ice cold 70% ethanol, dried and dissolved in 200 μ l of proteinase K digestion buffer (10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1 mM

EDTA, 0.5% v/v SDS). Proteinase K was added to 400 µg/ml and incubated at 50°C for at least 12 hours. The mixture was extracted with phenol/chloroform/isoamyl alcohol, and the resulting aqueous phase extracted with chloroform/isoamyl alcohol. The DNA was recovered as described in section 3.2.5 and resuspended in TE8.

Table 3.3. Embryonic stages collected during chinook salmon development. (1993 season, $11 \pm 1^\circ\text{C}$).

Number of days post-fertilisation	Stage No.	Description
1.2	7	Morula
3.3	8	Blastula
4	9	Start of gastrulation/epiboly
6	Late 9	Embryonic shield visible
11	16	Small fish-like embryo visible, tail bud free from yolk
15	19	Eye partially pigmented, 2/3 of yolk vascularised
26	22	Eyes fully pigmented, operculum covers first branchial slit
43	24	Just hatched

3.2.8 Quantification of DNA.

The concentration of DNA solutions was determined using the methods outlined in section 2.2.5.

3.2.9 Analysis of DNA.

The procedures for electrophoresis of DNA in preparation for Southern blotting and hybridisation, and PCR used in this chapter are essentially the same as outlined in sections 2.2.6 to 2.2.8. An exception is that the temperature profiling for the PCR was carried out using a Perkin Elmer Thermal Cycler 480.

3.2.10 Preparation of probes.

Probes were made by the random prime method using a Boehringer Mannheim kit. 25ng of probe DNA was denatured and incubated at 37°C for 30 minutes with 2 units of Klenow enzyme, 25 mM of each of the dNTPs; dTTP, dATP and dGTP and 50 mCi of [α - 32 P] dCTPs (specific activity 3000 Ci/mmol) (Amersham) in a total volume of 20 μ l random prime reaction buffer containing hexanucleotide primers. The unincorporated nucleotides were separated from the labelled probe, and the probe denatured prior to hybridisation, as described in section 2.2.9. The random primed probes produced had a specific activity of $1\text{--}2 \times 10^9$ cpm/ μ g.

3.2.11 Fry screening procedure.

DNA was extracted from fry and subjected to PCR analysis; pRSV-lacZ DNA was detected using the same primers described in section 2.2.6 and pSV2CAT DNA with the primers CAT1 and CAT2 (Figure 3.1) (see Appendix 1 for primer sequences and PCR conditions). The products were electrophoresed in 1.5% agarose gels in $0.5 \times$ TBE for 1.5 hours at 60V (40 mA) using a horizontal gel apparatus. The DNA was stained in 5 μ g/ml ethidium bromide and viewed under ultra-violet light (254 nm). The PCR products were then Southern blotted onto nylon membranes using the same technique outlined in section 2.2.8, with the exclusion of the depurination step. Membranes were hybridised to random primed probes that correspond to internal portions of the PCR products. A 166 bp fragment within the LacZ gene was amplified by PCR using primers Lac2 and LacZ (see Figure 2.1 and Appendix 1) and used for the detection of pRSV-lacZ DNA. The pSV2CAT probe was obtained by amplifying the pSV2CAT plasmid using the same primers as used for the fry screening, the primer binding sites were then removed by restriction endonuclease digestion (Figure 3.1) and the products separated by agarose gel electrophoresis. The probe was excised from the gel in a trough of low melting temperature agarose and used directly in a random prime labelling reaction or isolated from normal agarose using the Prep-A-Gene kit (BioRad).

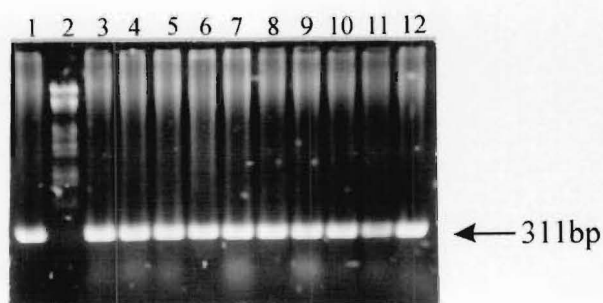


Figure 3.3. Example of the melanin concentrating hormone gene PCR products amplified from fry DNA samples. Lane 1: positive control, 311 bp MCH product amplified from salmon genomic DNA (0.2 μ g); 2: lambda DNA digested with *EcoRI* and *HindIII* (Fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 3 to 12: DNA extracted from salmon fry used as the template.

As a control, the 311 bp MCH fragment was amplified from at least 10% of samples from each series of extractions, as outlined in section 2.2.7. The MCH fragment was successfully amplified from all the crude fin extractions tested (Figure 3.3), indicating that amplifiable DNA was present in these samples.

3.3 Results.

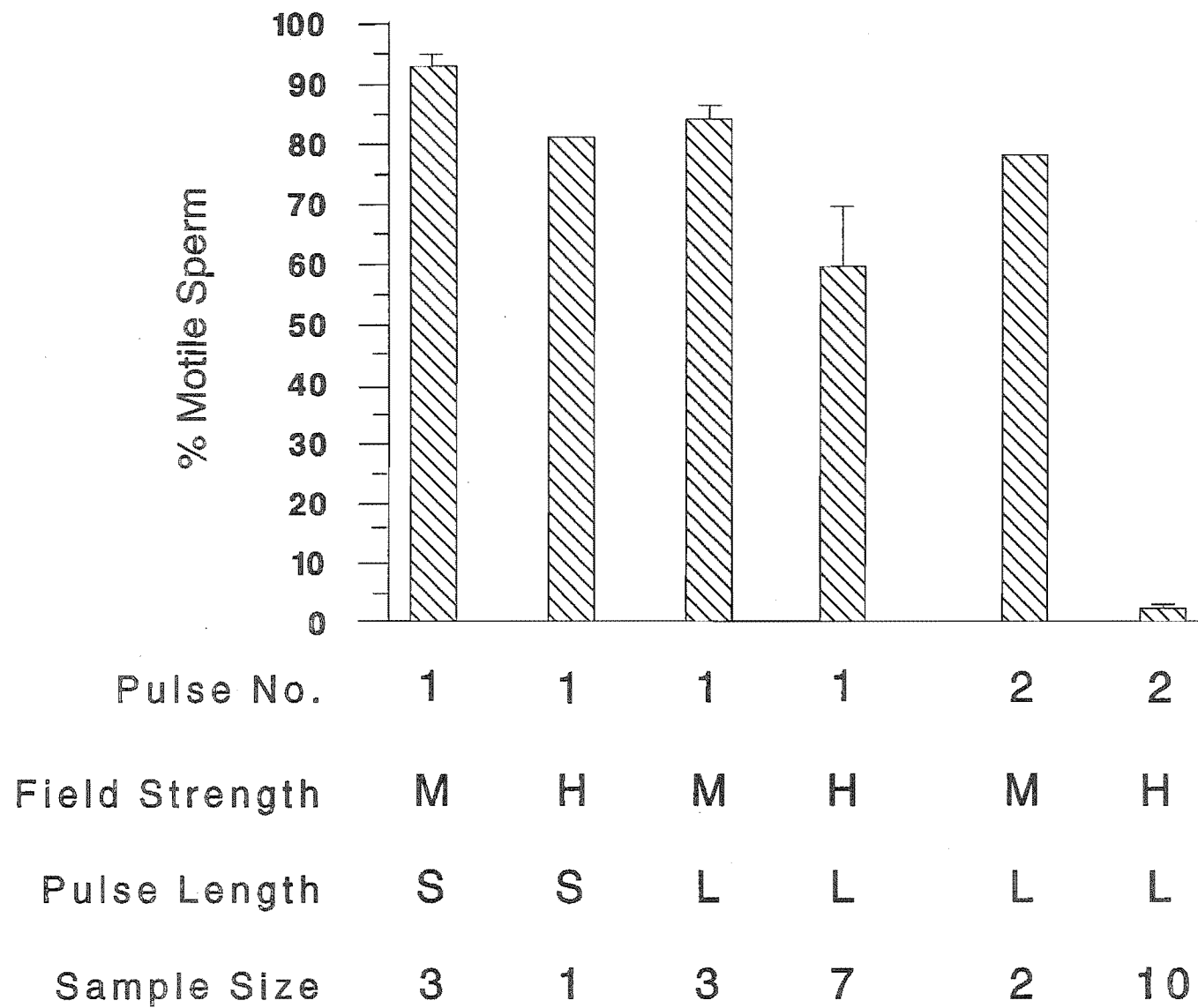
The results presented in this chapter cover experiments carried out in the chinook salmon spawning seasons of 1992 and 1993.

Data from the 1992 season, including the effect of the electroporation on the motility of the sperm and the relationship between this and survivorship to the fry stage, as well as gene transfer efficiencies, have been published (Symonds *et al.*, 1994b). This paper is included in Appendix 2. A preliminary study carried out using some of the 1993 season data has also been published (Walker *et al.*, 1995, see Appendix 2).

3.3.1 Sperm viability.

To determine the effect of electroporation on the fertilising capabilities of electroporated sperm, sperm motility and survivorship to the fry stage were analysed in detail in 1992. Several conditions were tested to determine the effect of the electrical and ionic parameters on the sperm sample: the field strength, pulse length, pulse number, and the ionic strength of the buffer. The motility of the sperm, after incubation on ice, was found to decrease with increasing field strength, pulse length, and number of pulses (Figure 3.4). When 2 pulses, each of 27.4 ms at 1000 V/cm, were applied, less than 5% of the sperm remained motile. The effect of applying up to 5 pulses of 18.6 ms or 27.4 ms, at 1000 V/cm, to the sperm was also tested. Motility directly after electroporation was found to decrease with increasing pulse number (Figure 3.5a and b); 5 pulses of 18.6 ms and 4 pulses of 27.4 ms reduced sperm motility to 0% (3 pulses of 27.4 ms resulted in a motility of 0.3%).

Figure 3.4. The effect of electroporation on sperm motility. Sperm were electroporated using a high ($H = 1000$ V/cm) or medium ($M = 625$ V/cm) field strength, with a single (1) or double pulse (2) of short ($S = 18.6$ ms) or long ($L = 27.4$ ms) duration. The number of individual sperm samples analysed is indicated (sample size).



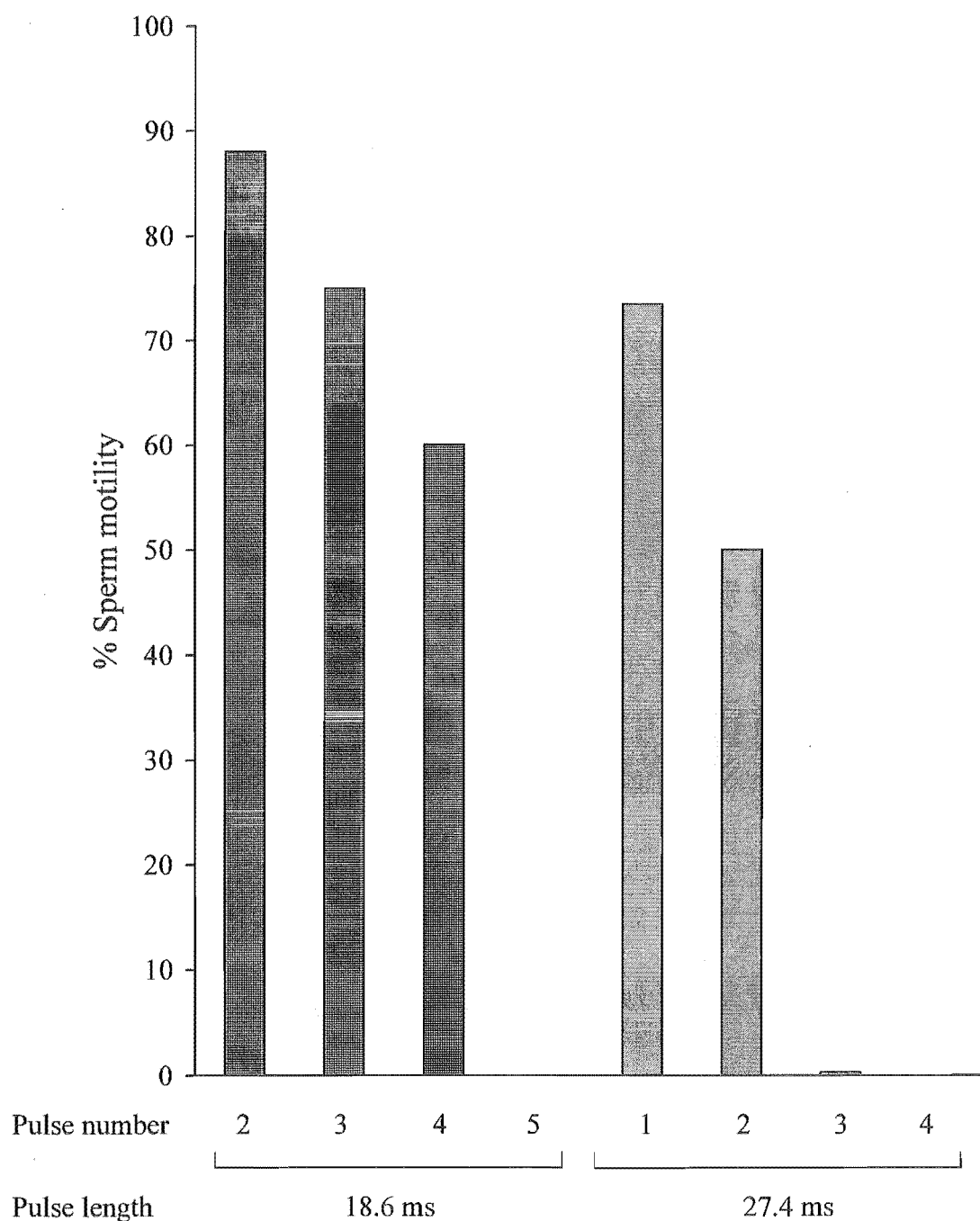


Figure 3.5 The effect of applying multiple electric pulses on the percentage of motile sperm directly after electroporation. Repeated pulses of 1000 V/cm of 18.6 ms (■) or 27.4 ms (□) were applied.

For all treatments 300 eggs were fertilised with electroporated sperm and the number of fry surviving to the 12 week stage is presented in Figure 3.6. In the 1992 season the eggs from three different females were used. As there was high mortality in the eggs from one of the females (female A), and survival was low irrespective of the electroporation conditions, these treatments were not included in the analysis. The number of eggs surviving to the fry stage decreased when 2 pulses were applied to the sperm, and was shown to be dependent on the percentage of motile sperm present in each sample used to fertilise 300 eggs ($r=0.69$, $p<0.01$) (Figure 3.7). Even when sperm motility was reduced to less than 5%, when 2 pulses of 27.4 ms at 1000 V/cm were applied, 100 fish out of a possible 300, survived to the fry stage.

Before any sperm were used for fertilisation the effect of the ionic strength of the electroporation buffer (HBS) on sperm motility was also determined. When the milt was diluted with $1 \times$ HBS in a ratio of 1:1 (final concentration of $0.5 \times$ HBS), the motility of the sperm was over 90%. However, when milt was diluted with $2 \times$ HBS in the same ratio, the motility was reduced to 40 to 50%. Therefore a final concentration of $0.5 \times$ HBS was used for most electroporations.

To determine if the inclusion of carrier DNA would enhance the transfer of plasmid DNA by electroporated salmon sperm, calf thymus DNA was added to the sperm/DNA mixture in 1 treatment. Prior to electroporation with 2 pulses of 27.4 ms at 1000 V/cm, sheared calf thymus DNA was added to a final concentration of 10 μ g/ml. In this treatment the motility after 10 minutes on ice was only 0.1% and 112 fry from 300 eggs fertilised survived to 12 weeks old.

To test the effect of increasing the time that the sperm/DNA solution was incubated on ice, sperm electroporated with 2 pulses at 1000 V/cm for 27.4 ms were incubated on ice for 30 minutes. The motility of this sperm sample was 0.25% and 177 fry survived to 12 weeks old when it was used to fertilise 300 eggs.

In comparison, when sperm were electroporated with 2 pulses at 1000 V/cm for 27.4 ms, without carrier DNA, and incubated on ice for only 10 minutes, the average sperm motility was 3.3% and an average of 103 fry survived to 12 weeks old (after 300 eggs were fertilised, $n=10$).

In 1993 the survival of the different treatments was not assessed.

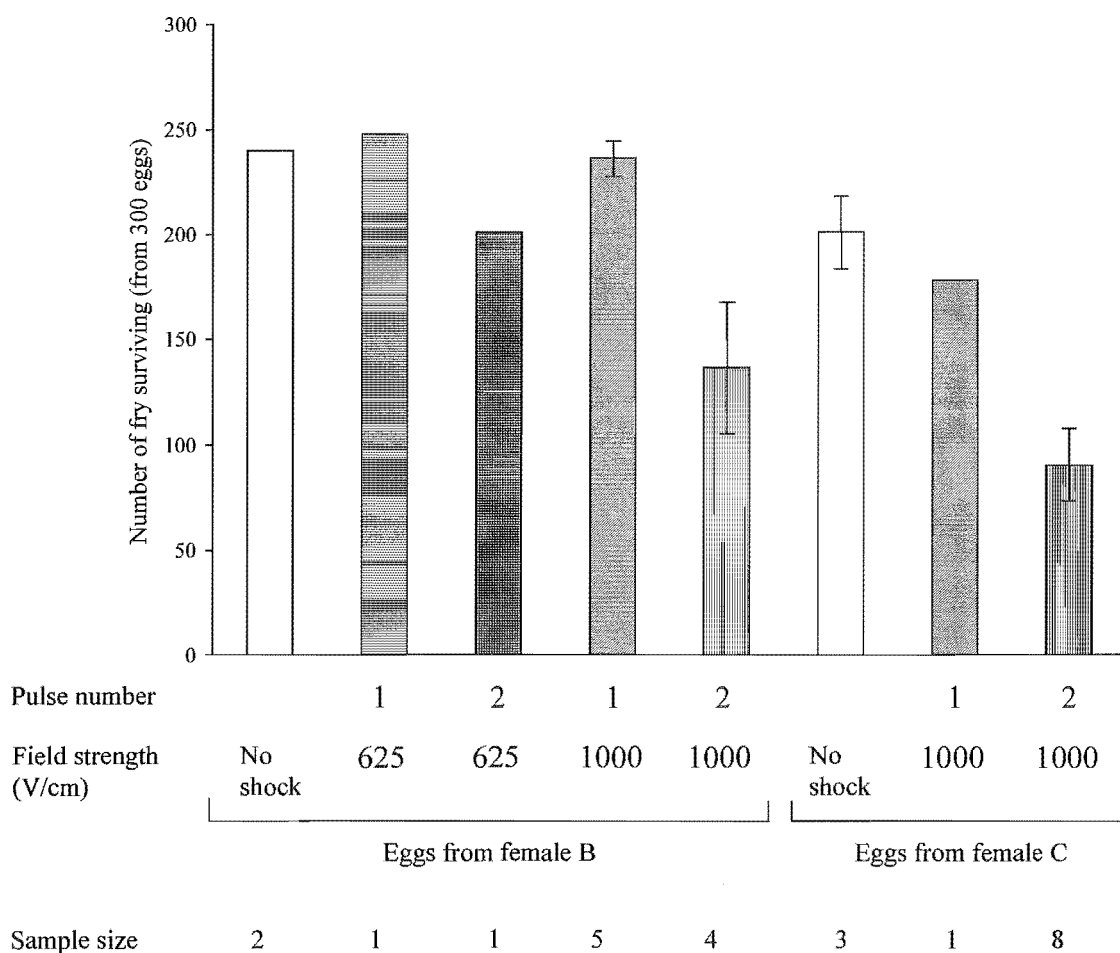


Figure 3.6. The effect of the sperm electroporation conditions on the number of fry surviving from 300 eggs (1992 season). The average number of chinook salmon surviving to the eyed stage resulting from 300 eggs (from female B and C) fertilised with normal sperm (No shock, □), sperm electroporated with one pulse of 625 V/cm (▤), 2 pulses of 625 V/cm (▥), 1 pulse of 1000 V/cm (▦), or 2 pulses of 1000 V/cm (▧) is shown. The number of individual groups analysed is indicated (sample size). In all the treatments presented a pulse length of 27.4 ms was used. Sperm electroporated with pulse length of 18.6 ms are not shown. These sperm were used to fertilise the eggs of female A. The mortality was high in all treatments and the number surviving was not related to the electroporation condition.

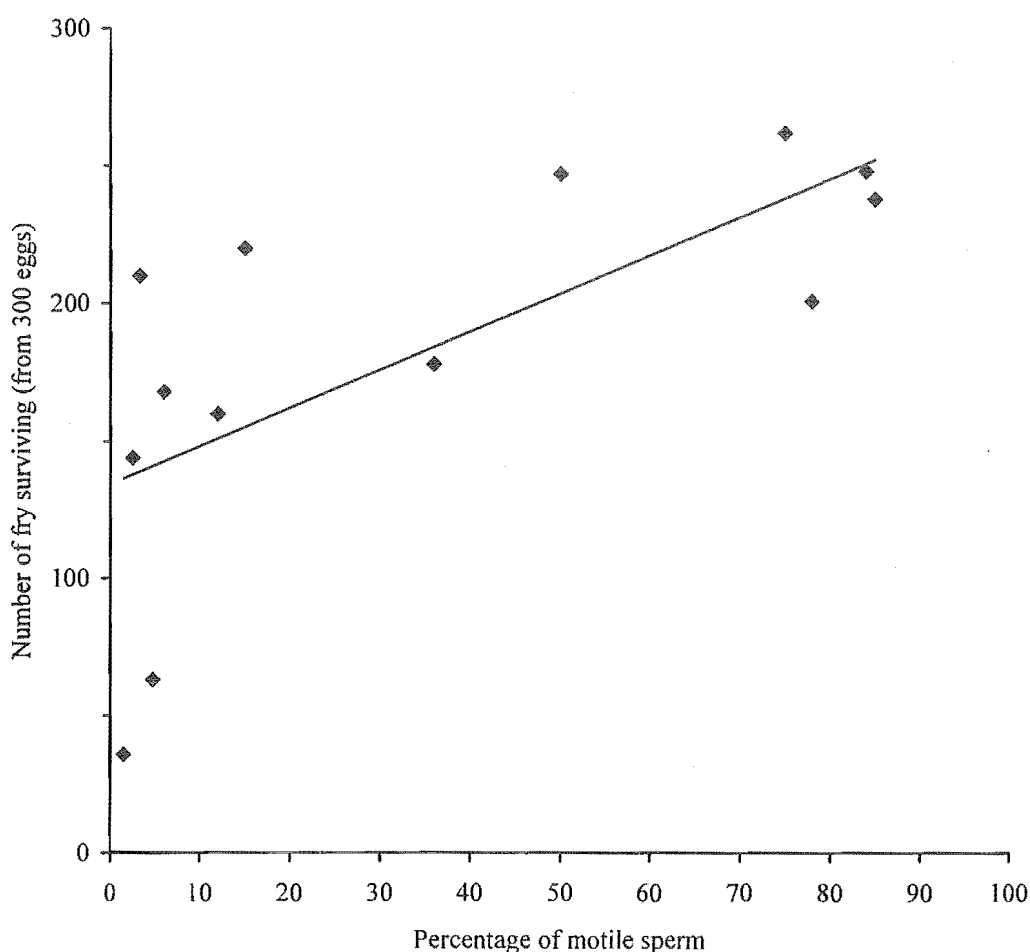


Figure 3.7. The number of fry surviving from 300 eggs as a function of the percentage of motile sperm present in the sample used for fertilisation. The motility was determined after the electroporated sperm were incubated on ice for 10 min. Only samples in which more than 1% of the sperm were motile were used for the analysis.

3.3.2 Effect of electroporation conditions on efficiency of gene transfer - 1992 fry.

The conditions tested and the gene transfer efficiencies obtained in the fry from the 1992 season are summarised in Table 3.1. Between 20 and 50 twelve week old fry from each treatment were screened by PCR for the presence of introduced plasmid DNA. Some of the PCR products generated during the screening could be visualised on agarose gels by ethidium bromide fluorescence, for example, see Figure 3.8. However, in most cases the product was only visible following Southern blot hybridisation, for example, see Figure 3.9.

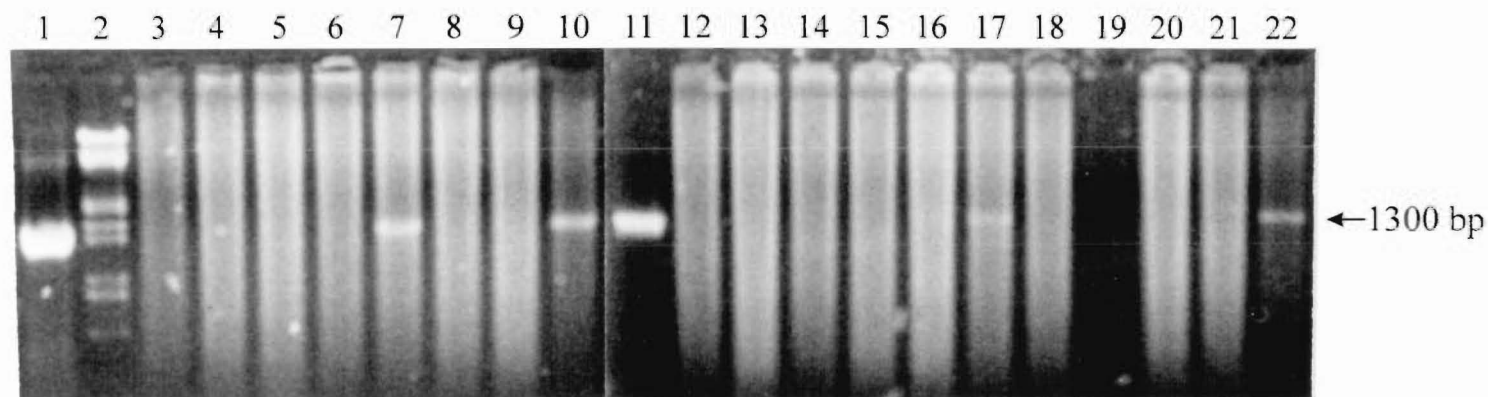


Figure 3.8. Detection of pRSVlacZ DNA in fry by PCR. Lanes 1 and 11: positive control, 1.3 kb product amplified from pRSV-lacZ DNA; 2: lambda DNA digested with *Eco*RI and *Hind*III (Fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); lanes 3 to 10: fry DNA from the 20 μ g/ml, 1000 V/cm, 18.6 ms, single pulse treatment; lanes 12 to 22: fry DNA from the 20 μ g/ml, 625 V/cm, 27.4 ms, single pulse treatment.

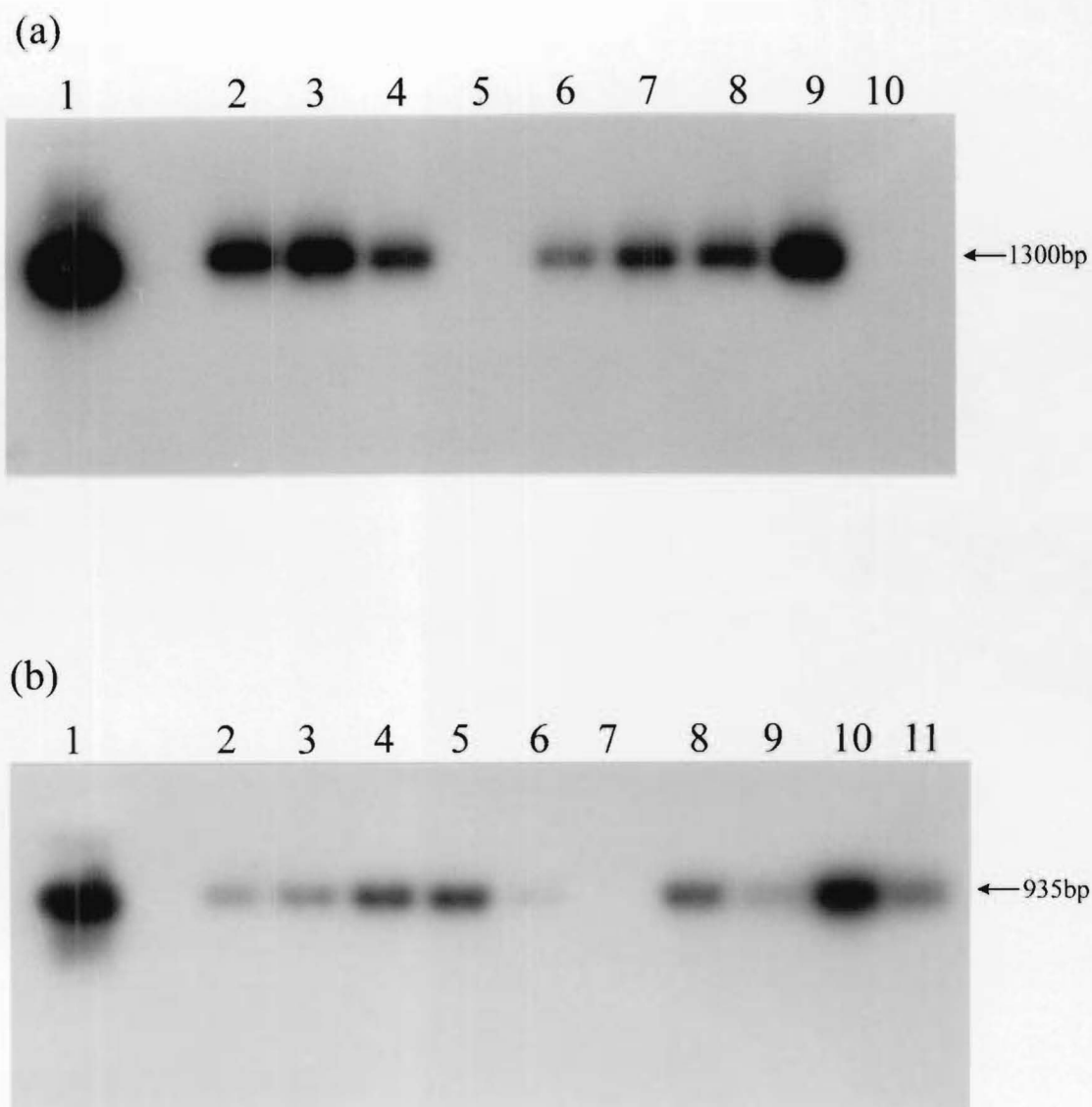


Figure 3.9. Detection of pRSVlacZ DNA in fry by PCR, followed by Southern analysis.

(a) Lane 1: positive control, 1300 bp product amplified from pRSV-lacZ DNA; 2 to 9: fry DNA from the 20 $\mu\text{g/ml}$, 1000 V/cm, 27.4 ms, double pulse treatment; 10: water control. All samples are from the same membrane, but lane 1 was exposed to X-ray film for 4 hours, and lanes 2 to 9 for 7 days.

(b). Lane 1: positive control, 935 bp product amplified from pSV2CAT DNA; 2 to 11: fry DNA from the 20 $\mu\text{g/ml}$, 1000 V/cm, 27.4 ms, double pulse treatment. All samples are from the same membrane, but lane 1 was exposed to X-ray film for 3 hours, and lanes 2 to 9 for 20 hours.

3.3.2.1 Transfer of pRSV-lacZ DNA.

No transfer of pRSV-lacZ DNA (circular or linear) was observed when non-electroporated sperm were used for fertilisations. In contrast, when electroporated sperm were used for the fertilisations up to 44% of the resulting fry contained the introduced DNA (Table 3.1). When 1 electric pulse was applied to sperm the highest gene transfer efficiencies (32% for linear DNA and 39% for circular DNA) were obtained at 1000 V/cm field strength and a pulse length of 18.6 ms. Increasing the duration of the pulse to 27.4 ms decreased transfer. However, a higher gene transfer efficiency was obtained when 2×27.4 ms pulses were applied. For example, when an electric field of 1000 V/cm was applied for 27.4 ms, 2 pulses resulted in the highest rate of gene transfer obtained, 44% (linear pRSV-lacZ), whereas 1 pulse gave only 5% gene transfer using the same DNA. Increasing the field strength of 2×27.4 ms long pulses from 625 to 1000 V/cm increased the efficiency of transfer of linear pRSV-lacZ DNA from 25% to 44% (see Table 3.1). No positive fry were detected when the sperm were electroporated at 625 V/cm with 2 pulses, 27.4 ms long, followed by a single 18.6 ms pulse, in the presence of circular pRSV-lacZ DNA.

3.3.2.2 Transfer of pSV2CAT DNA.

In contrast to pRSV-lacZ, pSV2CAT DNA was detected in 15% of the fry originating from sperm that had simply been incubated with the circular plasmid DNA. However, the efficiency of transfer of this plasmid was increased by the utilisation of electroporated sperm. When 1 pulse of 1000 V/cm and 27.4 ms was applied to sperm 28% of the fry were found to contain plasmid DNA. The application of 2 such pulses increased the gene transfer efficiency to 85% (Table 3.1).

3.3.2.3 Ionic strength of the electroporation buffer.

To test the effect of the ionic strength of the buffer on transfer efficiencies, some of the sperm electroporations ($625 \text{ V/cm} \times 18.6 \text{ ms}$, and $625 \text{ V/cm} \times 27.4 \text{ ms}$, single pulses only) were carried out with circular pRSV-lacZ DNA in a final concentration of $1 \times \text{HBS}$.

Transfer frequencies similar to those observed for $0.5 \times$ HBS were observed (Table 3.1). However, the highest gene transfer frequency (27%) was observed with the longer pulse length in $1 \times$ HBS, and the shorter pulse length in $0.5 \times$ HBS (17%).

3.3.2.4 Time of incubation at 0°C.

When the time the electroporated sperm were incubated on ice was increased from 10 to 30 minutes, using linear pRSV-lacZ and 2 pulses at 1000 V/cm for 27.4 ms, none of the 21 fry analysed were found to contain pRSV-lacZ DNA. In contrast when the incubation time of 10 minutes was used, 11 out of 44 fry (25%) were found to contain the plasmid DNA.

3.3.2.5 Carrier DNA.

When sheared calf thymus DNA was added, to a final concentration of 10 μ g/ml, to sperm incubated with linear pRSV-lacZ DNA, and prior to electroporation with 2 pulses of 27.4 ms at 1000 V/cm, none of the twenty fry tested were found to contain pRSV-lacZ DNA.

3.3.3 Effect of electroporation conditions on efficiency of gene transfer - 1993 embryos. (Table 3.4)

In 1993, embryos were collected from the treatments using 100 μ g/ml and 200 μ g/ml of plasmid DNA. PCR analysis of DNA extracted from embryos revealed that all sperm mixed with plasmid DNA (100 μ g/ml and 200 μ g/ml) were capable of transferring the DNA into a high proportion of embryos, irrespective of the electroporation conditions (no pulse, 1 pulse of 18.6 ms or 2 pulses of 27.4 ms) or plasmid DNA used. At all embryonic stages examined, up to 11 days post fertilisation, five out of five embryos tested using PCR were found to contain introduced plasmid DNA. There was 1 exception at the 4 day time point in the no pulse, 100 μ g/ml of circular pRSV-lacZ treatment, as only 4 out of the 5 individuals tested positive.

Table 3.4. Conditions and ages of embryonic samples taken in 1993 for PCR (P) and Southern analysis (S). The proportion of positives detected by Southern analysis is indicated. Sperm were electroporated with circular (C) or linear (L) DNA of pRSV-lacZ (LacZ) or pSV2CAT (CAT). The electroporation conditions were: no electroporation (0), 1 pulse 18.6 ms long of 1000 V/cm (1) and 2 pulses 27.4 ms long of 1000 V/cm (2).

Plasmid	DNA form	DNA concentration (µg/ml)	Electroporation condition	Number of days since fertilisation				
				1.2	3.3	4	6	11
LacZ	C	200	1	-	P S(0/5)	-	-	-
LacZ	C	200	2	-	P S(4/5)	-	-	-
LacZ	C	100	0	P S(0/5)	P S(0/5)	P	P	P
LacZ	C	100	1	P S(0/5)	P S(0/5)	P	P	P
LacZ	C	100	2	P S(3/5)	P S(0/5)	P S(0/5)	P S(0/5)	P S(0/5)
LacZ	L	200	1	-	P S(1/5)	-	-	-
LacZ	L	200	2	-	P S(4/5)	-	-	-
LacZ	L	100	0	P S(0/5)	P S(0/5)	P	P	P
LacZ	L	100	1	P S(1/5)	P S(0/5)	P	P	P
LacZ	L	100	2	P S(5/5)	P S(5/5)	P S(3/5)	P S(0/5)	P S(1/5)
CAT	C	200	0	P S(0/5)	P S(0/5)	-	-	-
CAT	C	200	1	P S(5/5)	P S(1/5)	-	-	-
CAT	C	200	2	P S(5/5)	P S(2/5)	-	-	-
CAT	L	200	0	-	P S(0/5)	-	-	-
CAT	L	200	1	-	P S(2/5)	-	-	-
CAT	L	200	2	-	P S(4/5)	-	-	-

The amount of product resulting from PCR amplification of the embryo DNA samples varied, and was dependent on the electroporation conditions (see Figure 3.10). In six of the nine occasions, where all three treatments were tested, increasing amounts of product were obtained in the zero, 1 and 2 pulse treatments respectively.

Plasmid DNA introduced into embryos by electroporated sperm was also detected by Southern analysis of the embryo genomic DNA samples. The frequency of gene transfer detected by this method is summarised in Table 3.4. The efficiency of gene transfer and the amount of the introduced DNA detected increased in the zero, 1 and 2 pulse treatments respectively. Figures 3.11 and 3.12 show autoradiographs that demonstrate this with the transfer of linear and circular pSV2CAT DNA.

3.3.4 Effect of electroporation conditions on efficiency of gene transfer - 1993 fry.

Fry, 12 weeks post-fertilisation, were screened for the presence pRSV-lacZ and pSV2CAT by PCR (Table 3.2). The results obtained were not as consistent as those obtained in 1992. However, some trends are evident. Overall, electroporated sperm were more efficient at transferring DNA into fry than non-electroporated sperm. In 9 out of 12 treatments where the application of a single (1×18.6 ms) or a double (2×27.4 ms) pulse was compared directly, the double pulse treatment gave a higher gene transfer efficiency. Additionally, the 2 pulse treatment gave the highest gene transfer frequency with both plasmids (33.3% and 46.7% for pRSV-lacZ and pSV2CAT respectively).

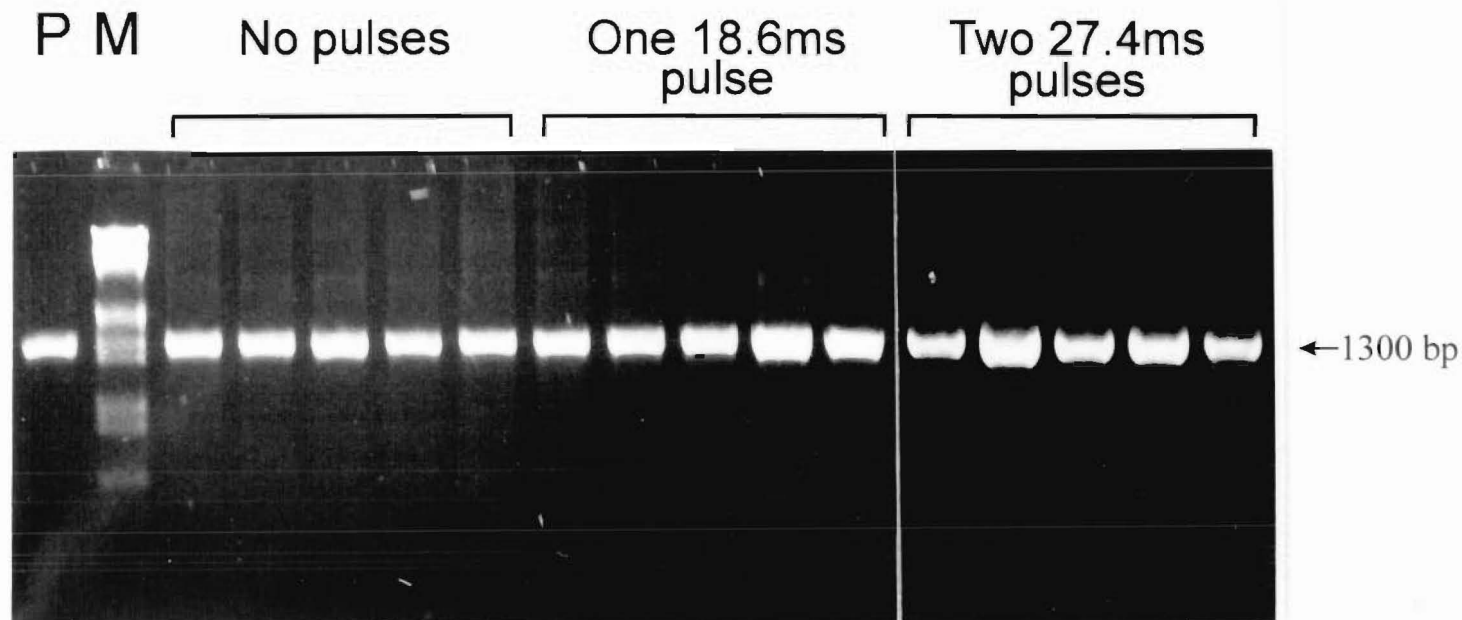


Figure 3.10. The effect of the electroporation conditions on the amount of plasmid specific PCR product obtained from embryo DNA samples. The electroporation conditions were no electroporation (No pulses), 1 pulse 18.6 ms long of 1000 V/cm (One 18.6ms pulse) and 2 pulses 27.4 ms long of 1000 V/cm (Two 27.4ms pulses). The embryos were 28 hours old and developed from sperm electroporated with 100 µg/ml of pRSV-lacZ DNA. A positive control of a 1.3 kb product originating from pRSV-lacZ DNA (P) is included. The second lane contains a molecular weight marker (M) DNA of lambda DNA digested with *EcoR* I and *Hind* III (Fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125).

Figure 3.11. Detection of linear pSV2CAT DNA in embryos by Southern analysis. DNA was extracted from 3.3 day old embryos resulting from sperm incubated with 200 µg/ml of linear pSV2CAT DNA. Lane 1: linear pSV2CAT DNA (100 pg); 2: linear pSV2CAT DNA (10 pg); 3: linear pSV2CAT DNA (1 pg); 4: 5 µg of salmon genomic DNA (the same DNA solution added to the embryo DNA samples to assist in efficient precipitation during the extraction procedure); 5 to 9: embryos resulting from un-shocked sperm (no shock); 10 to 14: sperm exposed to one 1000 V/cm × 18.6 ms pulse; 15-19: sperm exposed to two 1000 V/cm × 27.4 ms pulses.

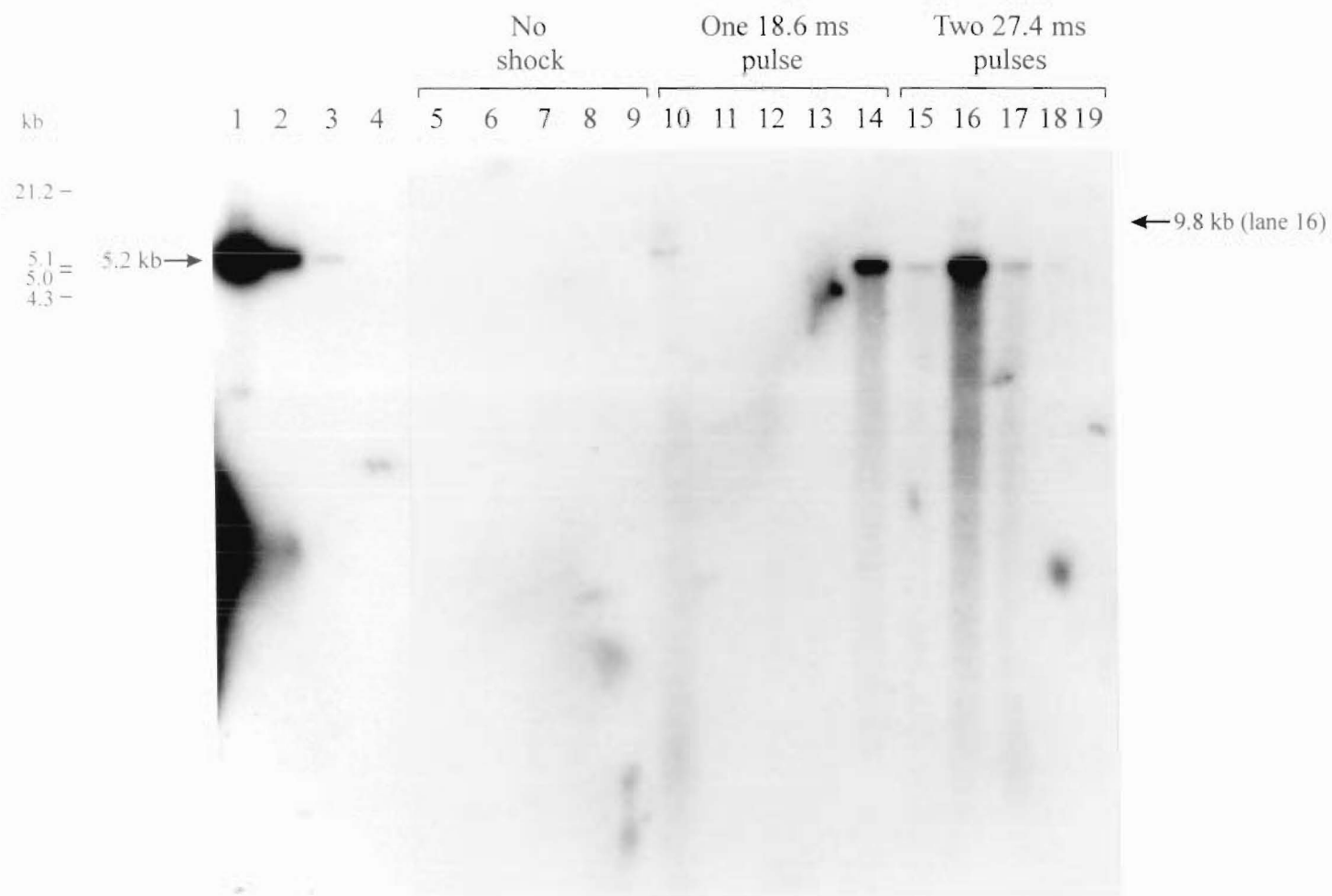


Figure 3.12. Detection of circular pSV2CAT DNA in embryos by Southern analysis. DNA was extracted from embryos resulting from sperm incubated with 200 µg/ml of circular pRSV-lacZ DNA. Lane 1: circular pSV2CAT DNA (100 pg); 2: circular pSV2CAT DNA (10 pg); 3: circular pSV2CAT DNA (1 pg); 4: 5 µg of salmon genomic DNA (the same DNA solution added to the embryo DNA samples to assist in efficient precipitation during the extraction procedure); 5 to 9: 1.2 day old embryos resulting from sperm exposed to one 1000 V/cm × 18.6 ms pulse; 10 to 14: 1.2 day old embryos resulting from sperm exposed to one 1000 V/cm × 18.6 ms pulse; 15-19: 3.3 day old embryos resulting from sperm exposed to two 1000 V/cm × 27.4 ms pulses. The positions of the nicked open circular (NC), covalently closed circular (CC) and supercoiled (SC) plasmid DNA are indicated.



3.3.5 Effect of different DNA constructs on gene transfer efficiency.

3.3.5.1 1992 fry.

In the 1992 season there were three occasions when similar treatments were used to transfer circular pRSV-lacZ and pSV2CAT DNA. A higher proportion of fry were found to contain pSV2CAT than pRSV-lacZ DNA by PCR (see Table 3.1), in addition the highest transfer efficiency obtained for pSV2CAT was 85% compared to 44% for pRSV-lacZ.

3.3.5.2 1993 embryos.

A comparison of embryos collected 3.3 days post-fertilisation and electroporated at 1×18.6 ms and 2×27.4 ms with 200 μ g/ml of circular and linear DNA of both plasmids, revealed that a similar proportion of individuals contained pRSV-lacZ and pSV2CAT. All embryos screened for the presence of both plasmids by PCR were found to contain the plasmid DNA. When screened by Southern analysis the introduced DNA of both types was detected in similar proportions of embryos (Table 3.4). For example, 1/5 and 4/5 positives were obtained in the 1×18.6 ms and 2×27.4 ms treatments respectively, when screening for linear pRSV-lacZ DNA, and 2/5 and 4/5 were obtained in the equivalent pSV2CAT treatments. However, more individuals containing circular pRSV-lacZ (4/5) than circular pSV2CAT DNA (2/5) in the 2 pulse treatment were detected, and in the 1 pulse treatment more were found to contain circular pSV2CAT (1/5) than circular pRSV-lacZ DNA (0/5).

3.3.5.3 1993 fry.

In 10 out of fifteen treatments, transfer frequencies were higher with pSV2CAT DNA than pRSV-lacZ (Table 3.2). This was especially true at the highest DNA concentration. For example, when sperm were exposed to 2 pulses of 1000 V/cm \times 27.4 ms, gene transfer efficiencies of 33.3 and 46.7% resulted for the transfer of circular and linear pSV2CAT DNA respectively, and 16.7 and 6.7% were obtained for the equivalent pRSV-lacZ treatments (Table 3.2).

3.3.6 Effect of DNA form (linear or circular plasmid DNA).

3.3.6.1 1992 fry.

DNA form did not have a noticeable effect on transfer as similar efficiencies were obtained with circular and linear pRSV-lacZ DNA (see table 3.2). For example, when 1 pulse of $625 \text{ V/cm} \times 18.6 \text{ ms}$ was applied, 17% and 25% of fry were found to contain circular and linear DNA respectively, and when 1 pulse of $1000 \text{ V/cm} \times 18.6 \text{ ms}$ was used, efficiencies of 39% and 32% resulted. Only circular pSV2CAT DNA was tested in 1992, so no comparison could be made for this plasmid.

3.3.6.2 1993 embryos.

The results in Table 3.4 show that more individuals containing linear than circular plasmid DNA were detected by Southern analysis of genomic DNA. In the $100 \mu\text{g/ml}$, 2 pulse, $1000 \text{ V/cm} \times 27.4 \text{ ms}$ treatments, pRSV-lacZ DNA was detected in 5 out of 5 embryos at 1.2 and 3.3 days old when linear DNA was used, while only 3 and 0 out of 5 were positive in the equivalent circular DNA treatments. A similar relationship was observed when circular and linear pSV2CAT DNA were used. For example, when $200 \mu\text{g/ml}$ of DNA was incubated with the sperm electroporated with 2 pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$, pSV2CAT was detected in 4 out of 5 embryos at 3.3 days old when linear DNA was used, while only 2 out of 5 tested positive when circular pSV2CAT DNA was used. On all 8 occasions where similar conditions were tested for both DNA types, and positive embryos were obtained, the proportion of positives was greater using linear DNA.

3.3.6.3 1993 fry.

Screening of fry by PCR revealed that, with one exception ($20 \mu\text{g/ml}$ with 2 pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$), linear pSV2CAT DNA was detected at the same or higher frequencies than circular pSV2CAT DNA (Table 3.2). For the 2 pulse, $1000 \text{ V/cm} \times 27.4 \text{ ms}$ treatments, gene transfer efficiencies of 16.7% and 46.7% were obtained when the sperm were incubated with 100 and $200 \mu\text{g/ml}$ of linear pSV2CAT DNA. When circular

pSV2CAT DNA was used for the equivalent treatments, 3.3% and 33.3% transfer obtained. In contrast to pSV2CAT, circular pRSV-lacZ DNA was detected at the same or with higher frequencies than linear pRSV-lacZ DNA, with one exception (20 µg/ml with 1 pulse of 1000 V/cm × 18.6 ms) (see Table 3.2). For example, when 2 pulses of 1000 V/cm × 27.4 ms were applied, gene transfer efficiencies of 33.3%, 16.7% and 16.7% (20, 100 and 200 µg/ml) were obtained when circular pRSV-lacZ DNA was incubated with the sperm, and when linear pRSV-lacZ was used only 13.3%, 6.7% and 16.7% of the fry were found to contain plasmid DNA.

3.3.7 DNA concentration.

3.3.7.1 1993 embryos.

In 1993 the effect of increasing the DNA concentration from 20, to 100 and 200 µg/ml was tested. Using PCR, greater amounts of product were amplified from 3.3 day old embryos when the concentration of the DNA incubated with sperm was increased from 100 µg/ml to 200 µg/ml. This was observed with circular and linear pRSV-lacZ DNA, for both 1 pulse of 18.6 ms and 2 pulses of 27.4 ms. Figure 3.13 shows an example of this effect when sperm were electroporated in the presence of circular DNA and shocked with 2 pulses of 27.4 ms.

Southern analysis showed that at 3.3 day old (DNA concentration comparisons were only made with pRSV-lacZ DNA and only at the 3.3 day stage) the number of individuals containing introduced DNA was low, and the relationship between DNA concentration and transfer efficiency was not clear (Table 3.4). However, 4 (3 faintly) individuals were found to contain pRSV-lacZ in the treatment originating from sperm incubated with 200 µg/ml of circular DNA and exposed to 2 × 27.4 ms pulses (Table 3.4 and lanes 10 to 14, Figure 3.14), whereas in the corresponding 100 µg/ml treatment, no introduced DNA was detected in the five individuals examined (Table 3.4 and lanes 15 to 19, Figure 3.14). A similar proportion of embryos tested positive with both DNA concentrations when linear DNA was used (Table 3.4).

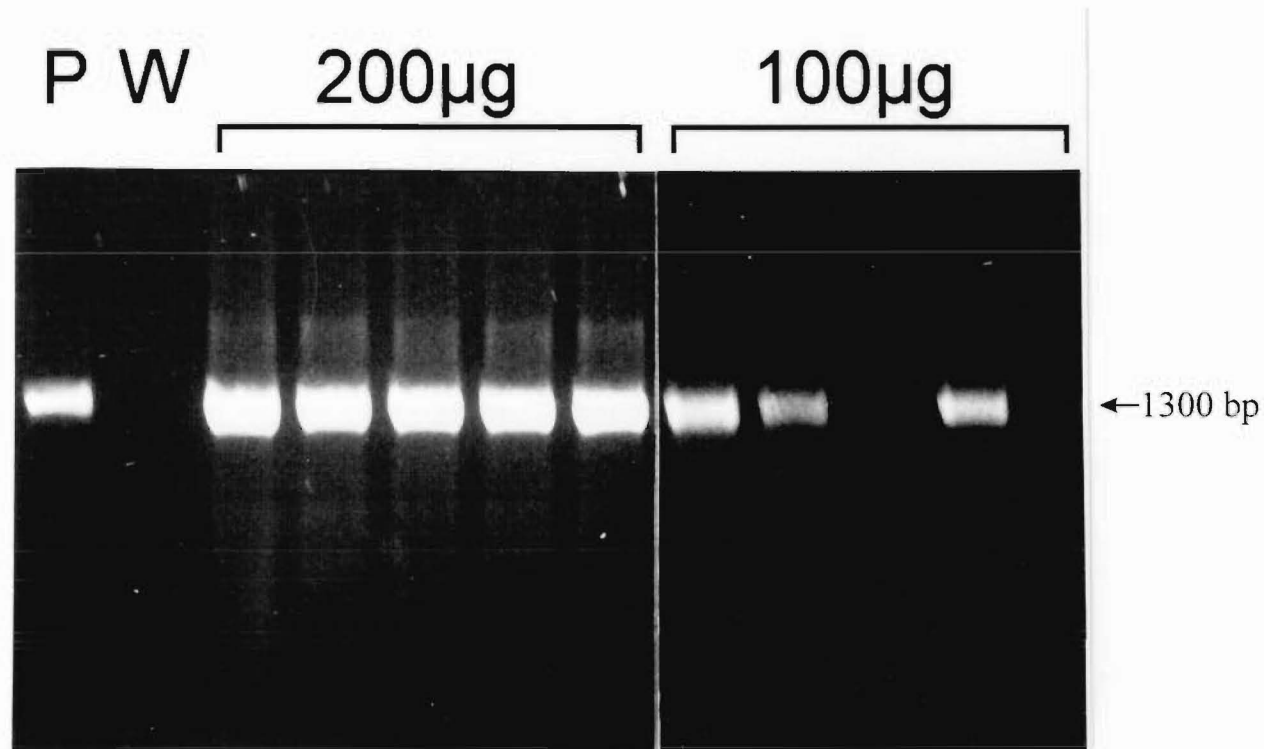
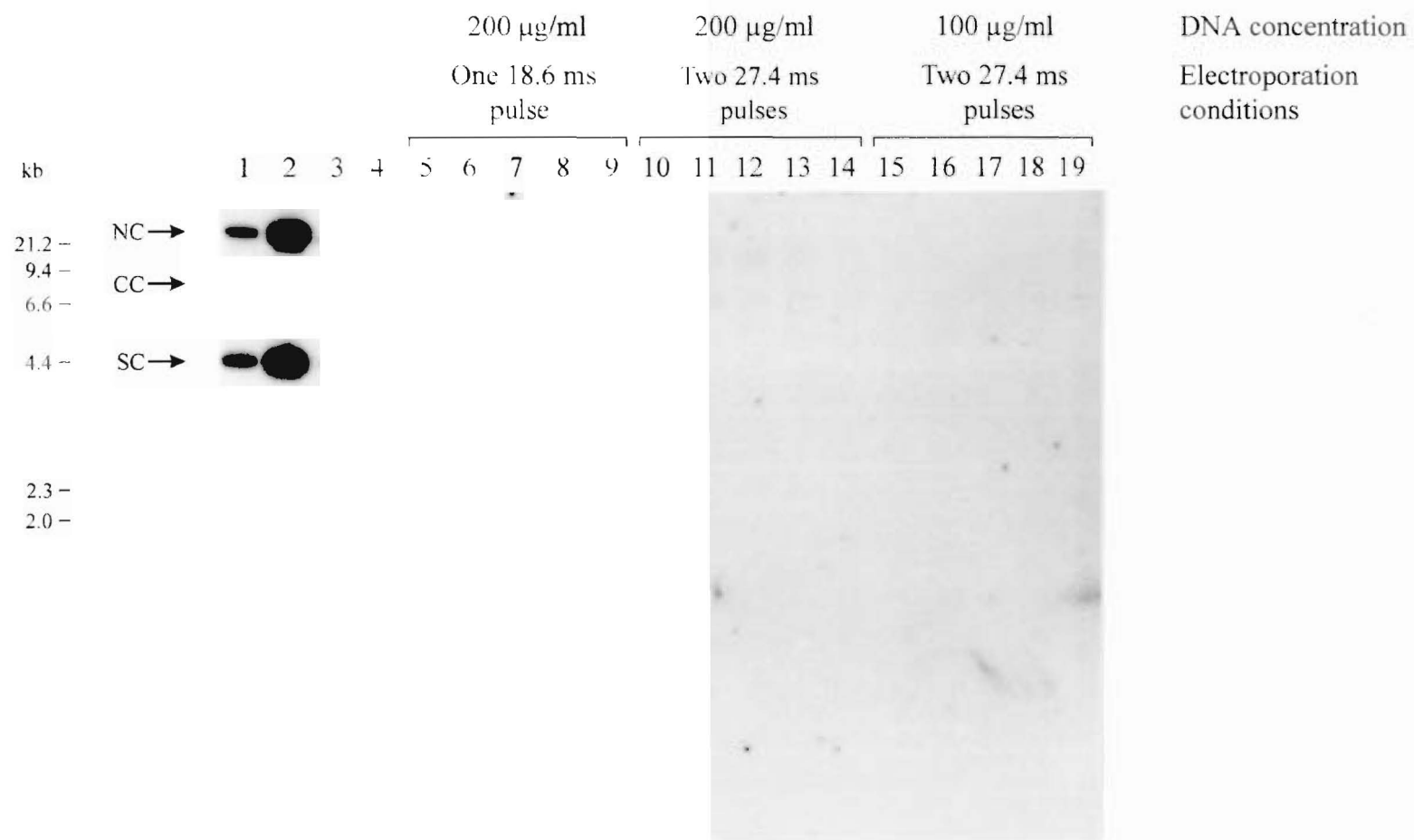


Figure 3.13. The effect of increasing the concentration of plasmid DNA electroporated with salmon sperm on the amount of amount of plasmid specific PCR product obtained from embryo DNA samples. Sperm were electroporated with 2 pulses 27.4 ms long of 1000 V/cm in the presence of circular pRSV-lacZ DNA (100 or 200 µg/ml). PCR analysis was carried out on blastula stage (3.3 days old) embryos. A positive control of a 1.3 kb product originating from pRSV-lacZ DNA (P) and a water control (W) are shown.

Figure 3.14. Detection of plasmid DNA in embryos resulting from sperm electroporated with 100 and 200 µg/ml of circular pRSV-lacZ DNA by Southern analysis. DNA was extracted from 3.3 day old embryos. Lane 1: circular pRSV-lacZ DNA (10 pg); 2: circular pRSV-lacZ DNA (100 pg); 3: circular pRSV-lacZ DNA (1 pg); 4: 5 µg of salmon genomic DNA (the same DNA solution added to the embryo DNA samples to assist in efficient precipitation during the extraction procedure); 5 to 9; embryos resulting from sperm electroporated with 200 µg/ml of DNA and exposed to one 1000 V/cm × 18.6 ms pulse; 10 to 14: embryos resulting from sperm electroporated with 200 µg/ml of DNA and exposed to two 1000 V/cm × 27.4 ms pulses; 15-19: embryos resulting sperm electroporated with 100 µg/ml of DNA exposed to two 1000 V/cm × 27.4 ms pulses. The positions of the nicked open circular (NC), covalently closed circular (CC) and supercoiled (SC) are indicated.



3.3.7.2 1993 fry.

PCR analysis of 12 week old fry (Table 3.2) indicated that although high gene transfer efficiencies can be obtained using the relatively low DNA concentration of 20 µg/ml, increasing the DNA concentration to 200 µg/ml is more likely to result in transfer. The highest gene transfer efficiency (of 47%) was obtained with the highest pSV2CAT DNA concentration. This was especially evident for linear pSV2CAT (Table 3.2); when 1 pulse of 1000 V/cm × 18.6 ms was applied gene transfer efficiencies increased from 3.3% to 6.7% and 36.7%, when the linear pSV2CAT DNA concentration was increased from 20 µg/ml to 100 µg/ml and 200 µg/ml respectively. Similarly for 2 pulses of 1000 V/cm × 27.4 ms, the efficiencies increased from 3.3% (20 µg/ml) to 36.7% (100 µg/ml) and 46.7% (200 µg/ml). A relationship between pRSV-lacZ DNA concentration and transfer efficiencies was not clearly evident, and the highest transfer of pRSV-lacZ (33.3%) was obtained using only 20 µg/ml (2 pulses of 1000 V/cm × 27.4 ms).

3.3.8 Southern analysis of fry DNA.

Fry genomic DNA samples that were positive by PCR screening were subjected to Southern analysis to determine if the transferred DNA could be detected by hybridisation analysis and, if so, to gain some information of its integration status. Genomic DNA extracted from 86 fry from the 1992 spawning season was analysed; 16 fry found to contain pRSV-lacZ DNA by PCR, plus 4 that tested negative; 26 positive for pSV2CAT DNA, plus 40 in which no pSV2CAT DNA was detected by PCR. Southern analysis was also carried out on 67 1993 fry genomic DNA samples previously screened by PCR; 32 pRSV-lacZ positive fry and 35 pSV2CAT positive fry. These represented various combinations of plasmid type, DNA concentration and DNA form. The number of fry screened from each treatment is shown in Table 3.5. No introduced DNA was detected by Southern analysis in any of the fry DNA samples.

Table 3.5. Number of 1993 season fry and treatments screened by Southern analysis of genomic DNA. Sperm were electroporated with circular (C) or linear (L) DNA of pRSV-lacZ (LacZ) or pSV2CAT (CAT). The electroporation conditions were 1 pulse 18.6 ms long of 1000 V/cm (1) and 2 pulses 27.4ms long of 1000 V/cm (2).

Plasmid	Conformation	DNA Concentration ($\mu\text{g/ml}$)	Electroporation Treatment	Number of fry screened
LacZ	C	20	2	4
LacZ	C	100	1	1
LacZ	C	100	2	5
LacZ	C	200	1	3
LacZ	C	200	2	5
LacZ	L	20	1	6
LacZ	L	20	2	5
LacZ	L	100	2	2
LacZ	L	200	2	2
CAT	C	200	1	11
CAT	C	200	2	7
CAT	L	200	1	7
CAT	L	200	2	10

3.4 Discussion.

Studies on the effect of electroporation on cell viability have demonstrated a trend of decreasing survival with increasing field strength and pulse length (Chu *et al.*, 1987; Shigekawa and Dower, 1988; O'Hare *et al.*, 1989; Weaver, 1993). The reasons for this increasing mortality are not clearly understood. A sudden membrane rupture may occur in some proportions of the cell membrane, leading to a large hole in the membrane, or alternatively, chemical imbalances, due to the passage of material through the pores, could cause disruption to the cellular processes (Weaver, 1993). Whatever the underlying mechanism, it seems that electroporated salmon sperm undergo a similar process. In 1992, the exposure of salmon sperm to electric pulses was found to decrease motility. The decrease was related to the electrical conditions, with the greatest reduction resulting when

multiple pulses of long duration and high field strength were applied to the sperm. These findings are consistent with previous results on the behaviour of electroporated salmon sperm, where motility was observed to decrease upon electroporation and the level was negatively related to the strength of the electric field applied (Sin *et al.*, 1993). The number of fish resulting from electroporated sperm was also shown to be related to the degree of motility and probably reflects a decrease in the viability and fertilising capabilities of sperm exposed to the electric field. However, the study of sperm viability indicated that relatively harsh electric pulses, which decrease sperm motility to less than 5% (approximately 2.5×10^8 motile sperm in 1 ml) will upon fertilisation of 300 eggs, still result in enough fish to make the use of this technique feasible.

Evidence of successful gene transfer by electroporated salmon sperm has been obtained over three seasons (1990 (Sin *et al.*, 1993), 1992 and 1993, this chapter). Gene transfer efficiencies of up to 47% being repeatedly achieved. These results indicate that the method is reproducible and therefore has the potential to be developed as an alternative gene transfer technique.

Although some transfer of pSV2CAT DNA occurred using non-electroporated sperm when DNA was simply mixed and incubated with milt, the exposure to electric pulses increased this transfer. Gene transfer by simple incubation of DNA with zebrafish sperm has also been reported (Khoo *et al.*, 1992; Patil and Khoo, 1996). However, electroporation of the sperm was shown to increase the efficiency of gene transfer in this species (Patil and Khoo, 1996). Additionally, when tested in common carp, African catfish and tilapia (Müller *et al.*, 1992), and in our previous experiments with salmon (Sin *et al.*, 1993) only electroporated sperm were found to transfer DNA. In the present study the detection of DNA in fry resulting from non-electroporated sperm may reflect the more sensitive screening method employed; PCR can detect down to femtogram amounts of DNA, well below that of Southern analysis, dot blot and expression analysis used in the other studies. Expression studies also rely on the construct being unmodified in the control and coding regions and not being inhibited by position effect after integration. Therefore the measured level of gene transfer may be higher using PCR as it relies only on the

presence of small sequences of DNA.

The highest field strength tested (the maximum possible with the equipment for the volume used), 1000 V/cm, proved to be the most successful at transferring DNA. This may reflect the small size of the cells being transformed, as the critical voltage necessary for the breakdown of a cell's membrane is inversely related to its diameter (Förster and Neumann, 1989). This is contrary to our earlier study (Sin *et al.*, 1993) where 625 V/cm appeared to represent a threshold over which no further increase in gene transfer efficiency was found. This difference could be due to the lower ionic strength of the buffer ($0.5 \times$ HBS) used in the present study to electroporate the sperm compared to the $1 \times$ HBS buffer used by Sin *et al.* (1993). Altering the ionic strength of the electroporation buffer is known to change the optimal voltage for electroporation (Andreason and Evans, 1988). Gagné *et al.* (1991) also found that buffer composition significantly affected plasmid retention by electroporated bovine sperm, and the difference between the buffers was dependent on the strength and duration of the electric field. There will be an upper level where the increased transfection of electroporated cells due to increased field strength is offset by cell death (for example, see Chu *et al.*, 1987). Although the use of higher voltages may increase transfer efficiencies, it is likely for salmon sperm that the level of mortality is nearing a limiting level at 1000 V/cm for salmon sperm using $0.5 \times$ HBS.

When other variables were kept constant, more efficient gene transfer was observed when double electric pulses were employed. Two pulses, 27.4 ms long, of 1000 V/cm gave the highest yields of transfer for both plasmids in 1992 and 1993. This combination of conditions also resulted in more DNA transfer into salmon embryos. This is in agreement with the observed increased sperm/DNA association when double electric pulses were applied to salmon sperm (Chapter 2). The increase in the gene transfer efficiency observed when two pulses were used may be due to either, an increase in the number of pores obtained upon the application of the second pulse, or further passage across the membrane through existing pores, as discussed in Chapter 2.

Including carrier DNA with the sperm/DNA mix did not lead to any increase in transfer efficiency and in fact no transfer of plasmid DNA into fry was detected when calf

thymus DNA was used. However, the sample size was small as only 1 group of 20 fry was screened. It is possible that the inclusion of carrier DNA inhibited gene transfer by competing with the plasmid DNA for sites of binding or internalisation on the sperm. Carrier DNA used in cell electroporation has been shown to be toxic to cells at high concentration (Chu *et al.*, 1987), but this was when 1000 µg/ml of DNA was used, significantly greater than the 10 µg/ml of the present study. The motility of the salmon sperm was reduced to 0.1% when carrier DNA was included. However, this was not below the range of motility for the equivalent treatment without carrier DNA, and 112 out of a possible 300 fish still survived to 12 weeks old. Evidence has been obtained that plasmid DNA does not become toxic to zebrafish sperm until levels are elevated to over 200 µg/ml (Khoo *et al.*, 1992). However, the possibility that sheared calf thymus DNA had a deleterious effect on the porated sperm in the sample at the level used, or alternatively that its inclusion could enhance gene transfer, cannot be ruled out, as no titration to determine the optimal level was carried out.

When the time the sperm were incubated on ice after electroporation was extended to 30 minutes, no plasmid DNA was detected in the resulting fry. The reason for this is unclear but may be due to increased mortality of the sperm in which pores had formed, due to a prolonged period of exposure to non-isotonic medium.

PCR analysis of fry from the 1992 season indicated that pSV2CAT DNA was transferred more efficiently than pRSV-lacZ DNA. The following season this pattern was again observed, although the difference was not as consistent. Because of the different sizes of the plasmids, the number of copies of DNA incubated with the sperm differed when the same concentration was used. The size difference equates to a ratio of 1.3:1.0 copies of pSV2CAT to pRSV-lacZ, which may explain the different efficiencies. Although both these plasmids have the same origin of replication there may be other differences between the plasmids that affect the efficiency of replication or persistence. DNA molecules as large as 150kb has been successfully introduced into cells via electroporation (Shigekawa and Dower, 1988). Therefore it seems unlikely that the relatively small difference in size of the two plasmids (pSV2CAT, 5185 bp; pRSV-lacZ

6900 bp) is responsible for their differential transfer. However, a study by Siguret *et al.* (1994) indicated that the efficiency of the transformation of *Escherichia coli* by electroporation is inversely proportional to the size of the DNA molecules over this size range (2.9 to 13.6 kb). Additionally, Ohse *et al.* (1995) found that the transformation efficiency of *Bacillus subtilis* by electroporation, in terms of transformants per microgram of DNA, decreased with increasing plasmid size. Therefore this factor may have contributed to the more efficient transfer of the smaller pSV2CAT DNA molecules. Another possible explanation is a disparate sensitivity of the PCR reactions used to detect the plasmids. However, amplification reactions, using dilutions of known amounts of each plasmid as template, indicated that both could be detected to a similar low level.

Studies of gene transfer by cell electroporation have found that linear plasmid DNA gives higher yields of stable transformation and expression (Potter *et al.*, 1984; Förster and Neumann, 1989). In this study there was evidence from the Southern analysis of embryonic DNA, that linear plasmid DNA was transferred more efficiently by electroporated salmon sperm than circular DNA. Although, the pSV2CAT PCR screening of fry in 1993 supported this, this pattern was not reflected in the overall results of fry age fish using PCR, and no significant difference was observed between the DNA forms. Additionally, the findings of the uptake study indicated that the amount of DNA associated directly with electroporated salmon sperm was not altered by its form (Chapter 2).

Studies of gene transfer into mice have demonstrated greater transfer efficiencies and integration rates using linear over circular DNA (Brinster *et al.*, 1985). Although several studies of gene transfer into fish, via microinjection through the egg chorion, have indicated that the same phenomenon occurs in fish (Chourrout *et al.*, 1986; Penman *et al.*, 1990), there is conflicting evidence and the advantage of using linear DNA requires further substantiation (see Fletcher and Davies, 1991, for a review). Iyengar and Maclean (1995) found higher expression and transfer efficiencies of linear DNA and hypothesised that this was due to more efficient concatemerisation of this form of DNA over circular molecules. For marketing and safety reasons, transgenic fish used in commercial aquaculture will undoubtedly require only the transfer of regulatory and coding sequences

of the gene to be introduced, and exclusion of the vector portions, especially any viral DNA or bacterial genes, will be mandatory. This will preclude the use of circular DNA. Therefore sperm electroporation conditions should be optimised for the linear form.

PCR and Southern analysis of embryo DNA revealed that increasing the DNA concentration to 200 µg/ml resulted in higher gene transfer frequencies. This was supported by the PCR screening of the 12 week old fry for pSV2CAT DNA (1993, Table 3.2) as transfer frequencies were found to be positively related to the DNA concentration. The same relationship was also observed when the efficiency of uptake of DNA by electroporated salmon sperm was examined (Chapter 2). These results are in agreement with other electroporation studies, as increasing the concentration of DNA present when cells are electroporated is known to increase the efficiency of transformation of mammalian cells (Chu *et al.*, 1987; Andreason and Evans, 1988; Anderson *et al.*, 1991). However, it should be noted that the pattern of transfer of pRSV-lacZ DNA into fry did not follow this relationship. While increasing the DNA concentration to yet higher levels may lead to better transfer, such levels may be toxic to the sperm as was reported for zebrafish sperm (Khoo *et al.*, 1992). The high amounts of DNA required for the procedure, especially if only the expression unit insert is to be used, may also constrain the use of higher concentrations.

Over two breeding seasons salmon sperm electroporation consistently resulted in gene transfer efficiencies of over 40%. These transfer efficiencies compare favourably with other studies on fish sperm electroporation (5 to 10%, Sin *et al.*, 1993; 2 to 4%, Müller *et al.*, 1992; 52%, Tsai *et al.*, 1995), embryo electroporation (4%, Inoue, 1990; 65%, Buono and Linser, 1992; 75%, Powers *et al.*, 1992; 48%, Müller *et al.*, 1993, 57%, Xie *et al.*, 1993; 70%, Zhao *et al.*, 1993; 15%, Tsai and Tseng, 1994), and embryo microinjection (for example, 75%, Chourrout *et al.*, 1986; 50%, Guyomard *et al.*, 1989; 25%, Culp *et al.*, 1991). In salmon relatively low gene transfer frequencies have been obtained following microinjection, for example, 4 to 6% in Atlantic and coho salmon (Fletcher *et al.*, 1988; Devlin *et al.*, 1994; Devlin *et al.*, 1995) and less than 1% in chinook salmon (Robert Devlin, personal communication; Jane Symonds, personal

communication). Although it is difficult to compare directly between any of these studies because of the different detection methods and developmental stages examined, the use of sperm for gene transfer in chinook salmon compares very well with the low rates of success obtained with microinjection in this species.

Although salmon sperm electroporation leads to effective transfer of plasmid DNA into salmon eggs, the level of introduced DNA is low in the fry stage salmon. In all fry examined, plasmid DNA could not be detected by Southern analysis of their genomic DNA, even though these fry were shown to be positive by PCR analysis. PCR is a very sensitive technique and is capable of detecting less than 1 copy of plasmid DNA per 300 fish cells. These observations suggest that the fry are mosaic, and that the levels of plasmid DNA in the fry are low. This observation is in contrast to previous results in chinook salmon, where introduced DNA was detected in fry stage fish by Southern analysis (Sin *et al.*, 1993). This discrepancy is difficult to explain as similar protocols were used in both studies.

3.4.1 Summary.

Overall, electroporated sperm have been shown to transfer exogenous DNA into embryos at frequencies higher than microinjection. The method has proved to be repeatable and optimal transfer conditions have been elucidated: 2 pulses of 1000 V/cm, 27.4 ms long, using 200 µg/ml of plasmid DNA. However, although electroporated sperm can be reliably used to transfer genes into salmon, the level of the introduced DNA in the resulting fish is low. Therefore, before the technique can be further improved, the fate and expression of the introduced DNA needs to be examined.

Chapter Four.

Fate and expression of DNA transferred into salmon by electroporated sperm.

4.1 Introduction.

In the production of transgenic fish it is important that the DNA introduced into the embryo persists, becomes stably incorporated into the genomic DNA of the host and is capable of being expressed. The understanding of what happens to the DNA after it has been introduced into the fish is crucial for the optimisation of any gene transfer technique. Therefore, in the development of a new technique, such as sperm electroporation, it is important to determine the sequence of events following the introduction of DNA by electroporated sperm.

Most studies of transgenic fish have only documented the persistence of DNA in free swimming or adult fish. The fate of DNA immediately after microinjection into one or two cell embryos has been traced in only a few species (Maclean *et al.*, 1987b; Stuart *et al.*, 1988; Chong and Vielkind, 1989; Winkler *et al.*, 1991; Volckaert *et al.*, 1994; Kozlov *et al.*, 1988; Zhu, 1992). There has been little study of the fate of DNA following sperm transfer.

The studies of plasmid DNA following microinjection have shown that the DNA is converted into alternate forms soon after injection, and is amplified during early development. When examined by Chong and Vielkind (1989) in medaka, modification of the plasmid DNA had occurred by the 32-64 cell stage (2 hours post-fertilisation) and this was followed by amplification of the DNA to a peak accumulation in the gastrula/neurula stage (1 day post-fertilisation). The maximum amount of DNA was approximately 10-12 times the 25pg originally injected. The amplified DNA was mainly found as high molecular weight concatemers. The modification was more pronounced when linear DNA was introduced, with all of it being converted to alternative forms (high molecular weight or supercoiled DNA) by the 32-64 cell stage. After blastulation only the high molecular weight form was detected. All possible forms of introduced circular DNA were initially present, including open circular, multimeric circular, supercoiled and the high molecular

weight forms. By the hatching stage (14 days post-fertilisation) only the high molecular weight form was detected in the medaka into which both the linear and supercoiled plasmid DNA had been introduced. The proportion of fish testing positive also decreased as the fish developed. The introduced DNA was apparently extrachromosomal and the amount declined as the fish developed.

Similar results to those found by Chong and Vielkind (1989) were also found in other studies in African catfish (Volckaert *et al.*, 1994), loach (Maclean *et al.*, 1987b; Kozlov *et al.*, 1988; Zhu, 1992), medaka (Winkler *et al.*, 1991) and zebrafish (Stuart *et al.*, 1988). Amplification of the injected DNA during early replication and the rapid modification of plasmid DNA into multimeric forms were common themes in these studies. The timing of the amplification, with respect to the developmental stage, and the subsequent decrease was similar between the different species. It was concluded that most of the transferred DNA existed extrachromosomally, was degraded as the fish developed and the proportion of fish containing such DNA decreased with age (Fletcher and Davies, 1991). Evidence from zebrafish embryos indicated that the levels of exogenous DNA and the length of time that it existed throughout development was positively influenced by the amount of DNA injected (Stuart *et al.*, 1988).

When linear DNA was transferred into loach and carp by the electroporation of fertilised eggs its fate during embryogenesis was similar to microinjected DNA (Xie *et al.*, 1993). The introduced DNA, detected by dot blot hybridisation, underwent amplification followed by degradation with a peak at the late gastrula stage.

Very few studies to date have addressed the fate of DNA transferred by sperm. In mammals there are only a few reports describing the detection of introduced DNA in adults (Lauria and Gandolfi, 1993), suggesting that DNA transferred by this method does not persist or become integrated. However, this can not be concluded until more detailed studies have been carried out. When sperm were used to transfer pSV2-gpt DNA into mice, the introduced DNA was detected by PCR in a high proportion of embryos from the 1 cell to the blastula stage (Hochi *et al.*, 1990). However, none of the 130 new born mice resulting from such embryos were found to contain the introduced DNA, while the

plasmid DNA was detected in 13 out of 146 mice that developed from microinjected embryos.

In a study by Müller *et al.* (1992), DNA transferred by electroporated sperm into carp, African catfish and tilapia, was detected by dot blot analysis of fry stage fish. Expression of the introduced plasmid DNA was observed in African catfish larvae, but no data on the fate or integration of DNA transferred by electroporated sperm into these species were reported. Evidence of persistence and inheritance of DNA transferred into zebrafish by incubation of DNA with sperm was found by Khoo *et al.* (1992). In a more recent investigation, DNA introduced into zebrafish by sperm incubation and electroporated sperm was detected in two week old fry, but no other assessment of the persistence or fate of the introduced DNA was carried out (Patil and Khoo, 1996). When DNA was transferred into loach using electroporated sperm, exogenous DNA was detected in 2 week and 6-month old fish by Southern analysis (Tsai *et al.*, 1995). Some evidence of the integration of the DNA into the genome was also observed, in the form of unique restriction fragments upon Southern analysis (Tsai *et al.*, 1995). Overall, the behaviour during embryogenesis of DNA transferred into any species by electroporated sperm has not been studied in detail. However, some studies do provide encouraging evidence that gene transfer by fish sperm can place exogenous DNA stably in the germline of fish species (Khoo *et al.*, 1992; Tsai *et al.*, 1995).

Most of the studies concerning the fate of introduced DNA have focused on the more easily studied warm water species such as medaka. However, a study by Guyomard *et al.* (1989) obtained evidence that linear DNA introduced into rainbow trout formed concatemers and that the introduced DNA persisted in this form in 12 month old fish. Linear and circular DNA microinjected into rainbow trout was also observed to form concatemers following transfer (Iyengar and Maclean, 1995). Overall, there are limited data from cold-water species such as salmonids, and little is known of the fate during development of the DNA transferred into these species. Development in cold-water fish is much slower than in tropical species such as medaka and zebrafish. The pattern of gene transfer efficiencies observed in later stages of development suggest that, similar to

warm-water fish, this group also gradually lose the introduced DNA over time, indicating that some DNA exists extrachromosomally in salmonids (Fletcher and Davies, 1991).

Evidence of transfer of DNA by electroporated salmon sperm was obtained as outlined in Chapters 2 and 3. The gene transfer efficiencies obtained in these studies, in terms of the proportion of fish containing the exogenous sequence, were high (up to 85%). However, it was clear that the amount of DNA in the salmon fry was low and the salmon were probably mosaic. In order to further optimise sperm electroporation, it is necessary to determine if this is due to the low levels of DNA transferred by the sperm and/or the fate of the DNA after introduction into the embryo. When microinjection is used to produce transgenic salmon pg amounts (10^6 to 10^7 copies) of DNA are introduced (Shears *et al.*, 1992; Devlin *et al.*, 1993). Salmon sperm contain approximately 3 pg of genomic DNA. Therefore it seems unlikely that a single sperm could transfer plasmid DNA into embryos at the same magnitude as a glass microneedle. However, the function of the sperm in delivering genomic DNA to the site of pronuclear fusion, may mean that, because of this more accurate placement, less DNA needs to be introduced into the eggs to produce the same outcome. It was the aim of the research presented in this chapter to determine the amount of DNA transferred by electroporated salmon sperm into the developing embryo, and examine its fate during embryogenesis.

An important step in any gene transfer study is to demonstrate that the introduced DNA has been integrated into the host genome. In many transgenic fish studies Southern analysis has been employed to demonstrate integration rather than extrachromosomal persistence of the DNA. The observation of one of two phenomena are used to argue that an integration event has occurred. The first is the co-migration with high molecular weight undigested genomic DNA. However, extrachromosomal DNA can have the same mobility as high molecular weight genomic DNA making inferences about the results of such blots difficult. The second is that, following digestion with appropriate restriction endonucleases, restriction fragments larger than expected for the gene used are obtained. However, such novel fragments could be caused by rearrangements of the DNA after transfer rather than by integration of the exogenous DNA.

The detection of the introduced DNA in the progeny of a transgenic individual is also not definitive evidence of integration. Extrachromosomal DNA has been observed in adult fish (Guyomard *et al.*, 1989; Winkler *et al.*, 1991) and the possibility in fish of the passage of such DNA to progeny has been noted (Fletcher and Davies, 1991; Khoo *et al.*, 1992). Evidence that inherited foreign DNA existed extrachromosomally in the progeny of zebrafish microinjected with plasmid DNA was obtained (Patil *et al.*, 1994). Such inheritance has also been observed in mice (Rassoulzadegan *et al.*, 1986) and nematodes (Stinchcomb *et al.*, 1985). Therefore to be certain of integration, non-mosaic progeny and a distribution of the transgene in the F₂ generation consistent with Mendelian inheritance must be shown (Fletcher and Davies, 1991). When examining plasmid DNA microinjected into trout eggs, Tewari *et al.* (1992) took the analysis of integration of introduced DNA a step further. The integration of the plasmid DNA into the genome, inferred by novel banding patterns on Southern blots and Mendelian inheritance patterns, was supported by the localisation of the introduced DNA on chromosomes by *in situ* hybridisation, providing much more convincing evidence of integration of the transgene.

An alternative method used to demonstrate integration of introduced DNA is the isolation and characterisation of a DNA fragment containing the junction between the introduced and genomic DNA. Ivics *et al.* (1993) attempted to determine the integration status of DNA introduced into zebrafish by using ligation-mediated PCR (LMPCR) to amplify putative junction fragments. Some evidence of integration, in the form of novel sized LMPCR products (containing plasmid DNA), was found using this technique, but as the nucleotide sequence of the fragments was not determined the possibility of rearrangements of the plasmid DNA, without integration into the genome, can not be ruled out.

It is not known whether the DNA transferred into salmon by electroporated sperm remains extrachromosomal in nature, or whether it is incorporated into the genome. Although the salmon are probably mosaic some DNA could be integrated in a small proportion of cells. Therefore it was important to determine whether integration had occurred following transfer. The options available to assess integration of the transferred

DNA were limited. No facilities existed to grow fish past the fry stage, therefore excluding the analysis of the F_1 and F_2 progeny. As the amount of transferred DNA in fish of appropriate developmental stages was too low to examine by Southern analysis, an amplification based technique to isolate junction fragments was more appropriate. Inverse PCR (IPCR) was used over LMPCR as the former involves a lower number of steps, so is theoretically more robust and sensitive, and has been used to successfully isolate junction fragments of integration events in transgenic mice (Ninomiya *et al.*, 1990; Hamada *et al.*, 1993) and mouse embryonic stem cells (Sutherland *et al.*, 1993).

In the assessment of any gene transfer technique it is necessary to demonstrate not only the persistence of the introduced DNA, but also the viability of the DNA, and its ability to be expressed. Therefore, to determine if the DNA transferred by electroporated sperm can be expressed, the enzyme products of the two reporter plasmids used, β -D-galactosidase (β -gal) and chloramphenicol acetyltransferase (CAT), were assayed in embryos. The plasmids pSV2CAT and pRSV-lacZ have been shown to express adequately in fish (for example, Liu *et al.*, 1990; Winkler *et al.*, 1991). The expression of the lacZ gene has been successfully detected in fish from a variety of constructs (eg. McEvoy *et al.*, 1988; Winkler *et al.*, 1991; Bayer *et al.*, 1992; Müller *et al.*, 1993). Histochemical staining using X-gal as a substrate is a common method in such studies (eg. Winkler *et al.*, 1991; Bayer *et al.*, 1992; Müller *et al.*, 1993) and a spectrophotometric assay has been used to detect β -gal activity in Atlantic salmon (McEvoy *et al.*, 1988). The CAT gene has been widely used in transgenic fish studies and its expression, which can be detected at low levels, is commonly detected by thin layer chromatography of the acetylated products of 14 C-chloramphenicol (eg. Chong and Vielkind, 1989; Stuart *et al.*, 1990; Winkler *et al.*, 1991; Yoshizaki *et al.*, 1992).

Apart from studies involving transient expression, the use of electroporated salmon sperm for gene transfer will only be practical if stable germline transformed lines can be produced. This must be preceded by the presence of a significant amount of introduced DNA early in the development of the embryo and integration of this into the genome. Therefore with these long term goals in mind the aims of the work presented in this

chapter were to determine, (1) the fate of DNA transferred into salmon by electroporated sperm, (2) the amount of DNA transferred, (3) its persistence in embryos and (4) the ability of the introduced DNA to be expressed.

4.2 Materials and Methods.

4.2.1 Sperm electroporation.

Embryos from several of the treatments set up in 1993 were sampled throughout development. Sperm electroporation was carried out as described in section 3.2.3. Combinations of plasmid type, DNA form, DNA concentration, and electroporation conditions (no shock, one pulse of $1000 \text{ V/cm} \times 18.6 \text{ ms}$, or two pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$) were set up and embryos were collected at different times throughout development (see Table 3.3, page 58, for details of the developmental stages). Table 4.1 shows the treatments and ages of the embryos sampled.

4.2.2 Detecting DNA in embryos.

The protocols for DNA extraction, PCR and Southern hybridisation analysis are outlined in sections 3.2.6 to 3.2.9.

4.2.3 Inverse PCR (IPCR).

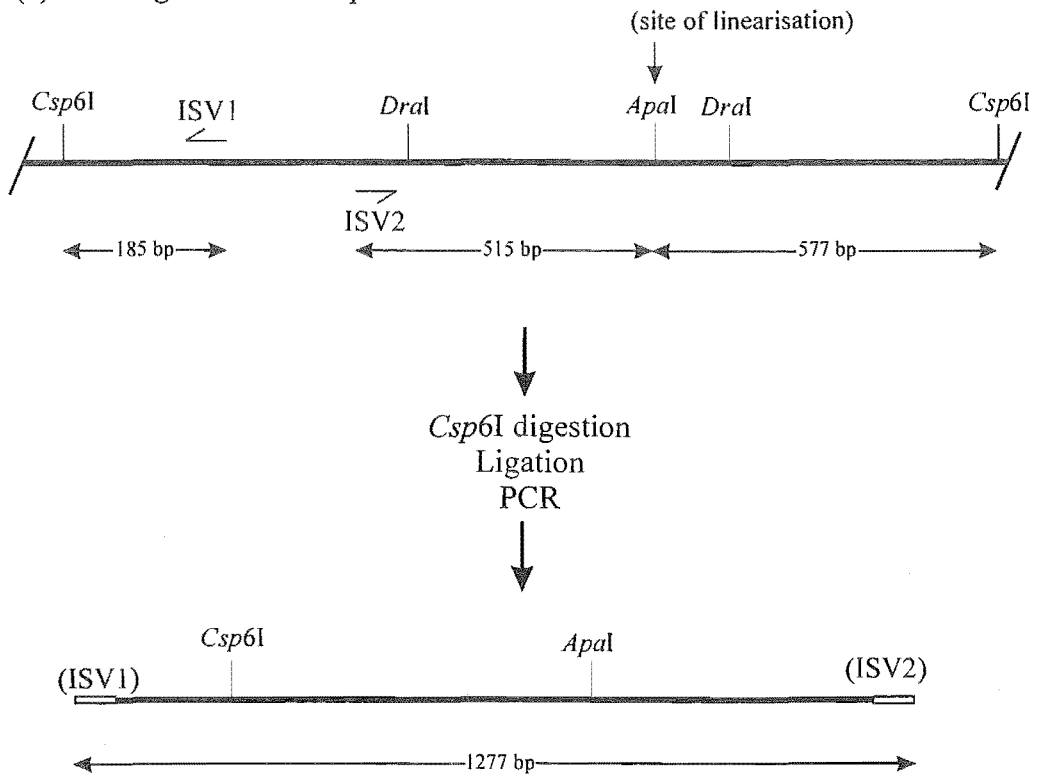
To test for the presence of integrated plasmid DNA in the fish, DNA amplification of putative junction fragments was attempted using IPCR. The procedure was based on that of Triglia *et al.* (1988) with modifications (Peter Davies and Andrew Catanach, personal communications). The technique involved restriction digestion of the salmon genomic DNA with an enzyme that cuts DNA frequently, and subsequent circularisation of the DNA. Two oligonucleotide primers which initiate polymerisation away from each other, near the site of linearisation, were used to amplify the region formed upon circularisation. If an integration event had occurred this would include the junction fragment (see Figure 4.1).

Table 4.1. Conditions and ages of embryonic samples taken in 1993 for PCR (P) and Southern analysis (S). The proportion of positives detected by Southern analysis is indicated. Sperm were electroporated with circular (C) or linear (L) DNA of pRSV-lacZ (LacZ) or pSV2CAT (CAT). The electroporation conditions were, no electroporation (0), 1 pulse 18.6 ms long of 1000 V/cm (1) and 2 pulses 27.4 ms long of 1000 V/cm (2).

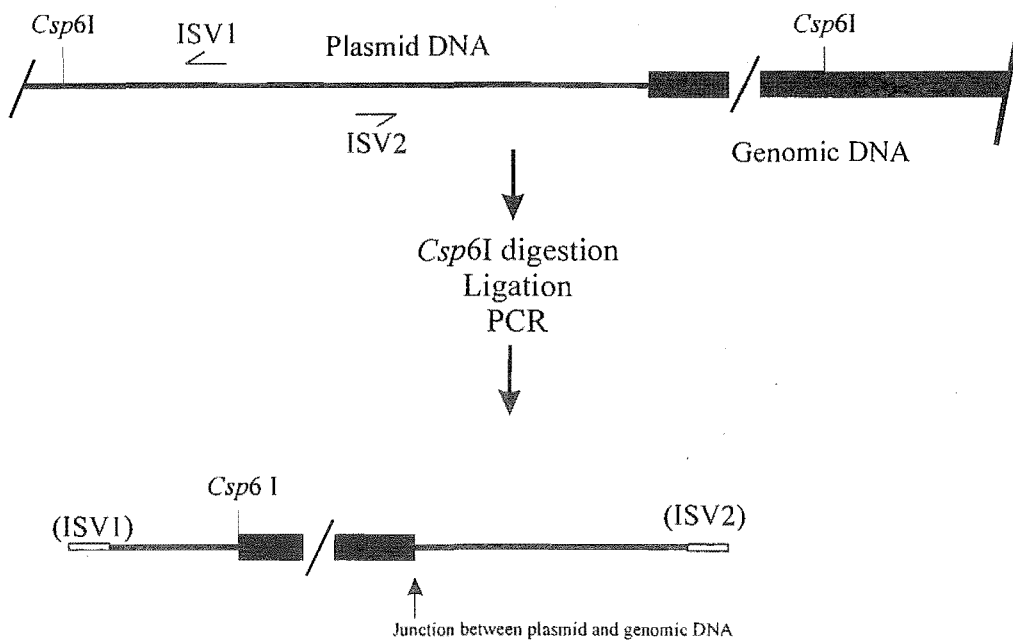
Plasmid	DNA form	DNA concentration (µg/ml)	Electroporation condition	Number of days since fertilisation				
				1.2	3.3	4	6	11
LacZ	C	200	1	-	P S(0/5)	-	-	-
LacZ	C	200	2	-	P S(4/5)	-	-	-
LacZ	C	100	0	P S(0/5)	P S(0/5)	P	P	P
LacZ	C	100	1	P S(0/5)	P S(0/5)	P	P	P
LacZ	C	100	2	P S(3/5)	P S(0/5)	P S(0/5)	P S(0/5)	P S(0/5)
LacZ	L	200	1	-	P S(1/5)	-	-	-
LacZ	L	200	2	-	P S(4/5)	-	-	-
LacZ	L	100	0	P S(0/5)	P S(0/5)	P	P	P
LacZ	L	100	1	P S(1/5)	P S(0/5)	P	P	P
LacZ	L	100	2	P S(5/5)	P S(5/5)	P S(3/5)	P S(0/5)	P S(1/5)
CAT	C	200	0	P S(0/5)	P S(0/5)	-	-	-
CAT	C	200	1	P S(5/5)	P S(1/5)	-	-	-
CAT	C	200	2	P S(5/5)	P S(2/5)	-	-	-
CAT	L	200	0	-	P S(0/5)	-	-	-
CAT	L	200	1	-	P S(2/5)	-	-	-
CAT	L	200	2	-	P S(4/5)	-	-	-

Figure 4.1. Outline of the inverse polymerase chain reaction (IPCR) procedure. DNA extracted from individuals shown to contain pRSV-lacZ DNA was digested with *Csp6I*, and following a ligation reaction, was used as the template in a PCR reaction with the primers ISV1 and ISV2. (a) If this is carried out on circular pRSV-lacZ, or *ApaI* digested pRSV-lacZ that has re-ligated at the *ApaI* site, a 1277 bp PCR product results. (b) If the *ApaI* linearised pRSV-lacZ plasmid DNA has been integrated into the salmon genome then the junction between the salmon genomic DNA and the pRSV-lacZ DNA is amplified. The length of the amplified fragment depends on the distance from the junction to the nearest *Csp6I* recognition site.

(a). Unintegrated circular plasmid DNA.



(b). Junction between integrated linear pRSV-lacZ plasmid DNA and salmon genomic DNA.



PCR product of novel length,
containing the junction between the plasmid and genomic DNA

Fish genomic DNA was digested to completion with the restriction enzyme *Csp6I*, which has a four base pair recognition site and leaves sticky ends. The restriction enzyme was then inactivated by incubation at 65°C for 10 minutes. 100 ng of the DNA was ligated with 3 units of T4 DNA ligase (Boehringer Mannheim) in 50 µl of ligation buffer (400 mM Tris, pH 7.5, 100 mM MgCl₂, 100 mM dithiothreitol, 100 mM adenosine triphosphate, 500 µg/ml bovine serum albumin), for 20 hours at 16°C. The DNA was then precipitated with ethanol and sodium acetate, incubated overnight at room temperature, centrifuged, washed and resuspended in 10 µl of TE8 as described in section 3.2.5. 2.5 ng of circular pRSV-lacZ DNA was similarly treated but 400 µl of TE8 was used. Prior to PCR, nicks were introduced into the template DNA by incubating it at 95°C for 20 minutes. Ten µl of the DNA solutions were used in PCR reactions as outlined in section 2.2.7, using the primers ISV1 and ISV2 (Figure 4.1, see Appendix 1 for primer sequences). The temperature profile used consisted of 4 minutes at 94°C followed by 31 cycles of 1 minute at 94°C, 1 minute at 58°C, and 2.5 minutes at 72°C. A final extension of 7 minutes was included. Half of the resulting PCR reactions were electrophoresed on 1% agarose gels, transferred to nylon membranes and hybridised to a *DraI* fragment of pRSV-lacZ that is specific to an internal portion of the IPCR product (see Figure 4.1), as outlined in sections 3.2.10 and 3.2.11. Some of the PCR products that resulted in bands of novel sizes upon autoradiography were further analysed. The remaining PCR reaction was electrophoresed and the region of the gel corresponding to the band of interest was excised. The DNA was then purified, using the method outlined in section 3.2.11., and used as a template for a further PCR reaction with the primers ISV1 and ISV2.

To test the efficiency of the enzyme digestion and ligation of the embryo genomic DNA, two primers, IPRL1 and IPRL2 (see Appendix 1 for primer sequences), were used which bind to the endogenous chinook salmon prolactin gene (Xiong *et al.*, 1992) and initiate polymerisation away from each other. PCR reactions using these primers were carried out on 10 (out of 25) of the samples analysed for integration of pRSV-lacZ with IPCR, after the *Csp6I* digestion and ligation. All of these produced the expected 416 bp fragment (Figure 4.2) indicating that the digestion and ligation reactions were successful. PCR reactions using un-ligated *Csp6I* restricted pRSV-lacZ and genomic DNA as a template did not yield the expected 1277 and 416 bp bands (using the primer pairs: ISV1 and ISV2; IPRL1 and IPRL2), demonstrating the dependence of these bands on the IPCR process (Figure 4.2).

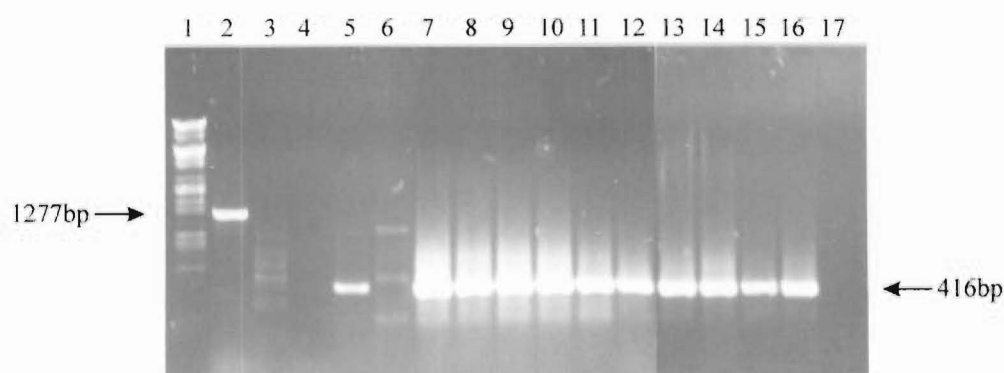


Figure 4.2 Inverse PCR control reactions. Lane 1: lambda DNA digested with EcoR I and Hind III (Fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125). Lanes 2 to 4: PCR reactions with the primers ISV1 and ISV2. Lanes 5 to 17: PCR reactions with the primers IPRL1 and IPRL2. Lane 2: circular pRSVlacZ cut with *Csp6* I and ligated; 3: circular pRSVlacZ cut with *Csp6* I with no ligation; 4: water control; 5: control chinook salmon genomic DNA cut with *Csp6* I and ligated; 6: control chinook salmon genomic DNA cut with *Csp6* I with no ligation; 7 to 16: genomic DNA extracted from test embryos or fry, cut with *Csp6* I and ligated; 17: water control.

4.2.4. β -D-galactosidase (β -gal) expression.

4.2.4.1 Histochemical assay using X-gal.

This technique is based on the hydrolysis of 5-bromo-4-indolyl- β -D-galactoside (X-gal) by β -gal to generate galactose and soluble indoxyl molecules, which are then oxidised to insoluble indigo (MacGregor *et al.*, 1991).

β -gal was assayed histochemically using the method described by Müller *et al.* (1993). This was carried out at pH 8 as strong endogenous activity can be observed in the guts and yolk at pH 7.5 and less. Embryos were washed twice in phosphate buffer (0.1M, pH 8) and then fixed in the same buffer containing 0.2% glutaraldehyde, 2 mM MgCl_2 and 5 mM ethylene glycol-bis-tetraacetic acid (EGTA). The fixative was removed by washing three times in phosphate buffer containing 2 mM MgCl_2 , 0.02% Nonidet P-40 and 0.01% sodium-deoxycholate. Staining at 37°C overnight was carried out in the same wash buffer containing 25 mM potassium ferricyanide, 25 mM potassium ferrocyanide and 1 mg/ml X-gal. The embryos were then washed twice in phosphate buffer with 3% dimethyl sulfoxide, three times in 70% ethanol and then stored under 70% ethanol. Purified β -gal (BioRad) was used as a positive control. The embryos were screened for the presence of any blue staining by viewing with a binocular light microscope with up to 400 \times magnification.

4.2.4.2 Spectrophotometric assay using *o*-nitrophenol- β -D-galactoside (ONPG).

β -gal converts ONPG to galactose and the chromophore *o*-nitrophenol, which can be detected by measuring the absorbance at 420 nm (MacGregor *et al.*, 1991).

The procedure used for this assay was based on the method described by MacGregor *et al.* (1991). Individual frozen embryos were thawed in ice cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM monopotassium phosphate), dissected with watch makers forceps into this buffer and the chorion removed. The cells were recovered by centrifugation at 3,400 rpm (Jouan 14.11) for 5 minutes at 4°C and removal of the supernatant. The cells were washed twice by the addition of 1.5 ml of cold PBS followed by centrifugation and supernatant removal as

before. Following a final wash in 1.5 ml of cold TEN (40 mM Tris-HCl, pH7.5, 1 mM EDTA, 150 mM, NaCl) the pellets were resuspended in 30 μ l of cold 0.25 M Tris-HCl, pH8. The cell suspension was then subjected to three rounds of freeze thawing, using liquid nitrogen and a 37°C water bath, with vortexing after each thaw cycle, and centrifuged at 12,000 rpm (Jouan 14.11) for 5 minutes at 4°C. The resulting supernatant was made up to 100 μ l with distilled water and 100 μ l of 2 \times β -gal assay buffer (200 mM sodium phosphate, pH7.3, 2 mM MgCl₂, 100 mM β -mercaptoethanol, 1.33 mg/ml ONPG) added. After incubation at 37°C for three hours 333 μ l of 1 M sodium carbonate was added, the colour noted and the OD₄₂₀ measured. If β -gal is present a yellow colour should become apparent (MacGregor *et al.*, 1991). The OD₄₂₀ reading obtained in the test embryos was compared to the OD₄₂₀ obtained in negative control embryos. This allowed correction for endogenous activity and absorbance from sources other than the end product. The maximum absorbance obtained in the control embryos was subtracted from the sample OD₄₂₀ readings. The level of β -gal was determined using a standard curve (Figure 4.3) plotted from OD₄₂₀ absorbances obtained from a dilution series of purified β -gal (BioRad). One unit of β -gal will hydrolyze 1.0 μ mole of *o*-nitrophenol beta-D-galactoside to *o*-nitrophenol and D-galactose per minute at pH 7.3 and 37°C.

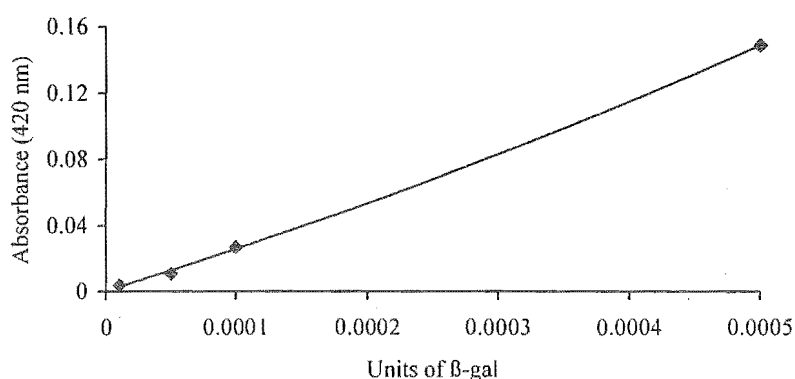


Figure 4.3. Standard curve for the level of β -D-galactosidase activity determined by the conversion of *o*-nitrophenol- β -D-galactoside to *o*-nitrophenol.

4.2.5 Chloramphenicol acetyltransferase (CAT) expression.

This assay detects the presence of the CAT enzyme, and relies on its acetylation of chloramphenicol to form 1-acetate chloramphenicol and 3-acetate chloramphenicol (1,3-diacetate chloramphenicol may also form when CAT is present at high concentrations) which, because of their higher mobilities through organic solvents, can be separated from the non-modified form by chromatography (Gorman *et al.*, 1982).

The cell extracts and acetylation reactions were carried out using a Promega Kit-"CAT Enzyme Assay System With Reporter Lysis Buffer". Individual frozen embryos were thawed in ice cold PBS, dissected with watch makers forceps into this buffer and the chorion removed. The cells were recovered by centrifugation at 3,400 rpm (Jouan 14.11) for 5 minutes at 4°C, and the supernatant was removed. The cells were then washed twice by the addition of 1.5 ml of cold PBS followed by centrifugation and supernatant removal as before. The tissue was homogenised in 50 µl of Reporter Lysis Buffer (Promega-proprietary information) followed by an incubation at 60°C for ten minutes to inactivate endogenous deacetylase activity. The homogenate was then centrifuged at 12,000 rpm (microfuge) for two minutes and the supernatant removed. Approximately 50 µl of the cell extracts were used in acetylation reactions with 10 µl of 0.025 mCi/ml ¹⁴C-chloramphenicol (Amersham; 54 mCi/mmol, 200 Ci/ml), 5 µl of n-butyryl Coenzyme A made up to 125 µl with distilled water. Positive (0.1 unit of CAT in 5 µl; one unit will convert 1.0 nmole of chloramphenicol and acetyl-CoA to chloramphenicol 3-acetate and CoA per minute at pH 7.8 and 25°C) and negative control reactions, plus dilutions, were similarly set up. Reactions were carried out at 37°C for 20 hours and then stopped by the addition of 500 µl of ethyl acetate. Each sample was vortexed for one minute and then centrifuged at 12,000 rpm (microfuge) for three minutes. The organic phase was then removed and evaporated to dryness. The resulting residue was resuspended in 15 µl of ethyl acetate and then spotted onto a silica gel TLC plate (J.T. Baker) that had been equilibrated with chloroform/methanol (97:3). Chromatography was then carried out in the same solvent until the front had migrated approximately 150 mm. The plate was then dried and exposed to X-ray film (Amersham Hyperfilm) with intensifying screens for 28 days at -80°C. The presence or absence of CAT activity in the samples was determined

by comparing the signals obtained in sample lanes with those obtained for the positive and negative controls.

4.3 Results.

The results presented in Chapter 3 covered the gene transfer efficiencies obtained in 1992 and 1993 using a range of electroporation conditions. The same 1993 treatments were also analysed for the persistence and expression of the introduced DNA in embryos and the results are presented in this chapter. Consequently there is some overlap of the results and some of the figures presented in Chapter 3 are also used in this chapter to illustrate the persistence of DNA in embryos. Therefore to avoid duplication the reader is directed to the relevant tables, figures and page numbers in Chapter 3 when appropriate.

4.3.1 Fate of the DNA: Persistence of plasmid DNA in salmon embryos.

When morula stage embryo DNA samples were analysed by Southern hybridisation high levels of introduced DNA were found to be present. For example, Figures 4.4 and 4.5 show that over 100 pg, 1×10^7 copies, of both circular and linear pRSV-lacZ were detected in 28 hour old embryos (resulting from sperm treated with 2 electric pulses of 1000 V/cm, 27.4 ms long). The amount of DNA was estimated from the intensity of the bands relative to those of the plasmid dilution series. As the age of the sampled embryos increased the amount of DNA detected, as well as the proportion of embryos testing positive decreased (Table 4.1). A distinct drop off in the level of plasmid DNA and proportion of positive embryos was observed between the 28 and 80 hour post-fertilisation time points. For example, Figure 3.12 (page 75) shows results of the Southern analysis of embryos from the circular pSV2CAT treatments. Five out of five embryos tested positive at 1.2 days compared to two out of five at 3.3 days in the two pulse treatment. In the one pulse treatment the proportion of positives decreased from four out of five to one out of five between these two embryonic stages. The overall decrease in the proportion of embryos found to contain plasmid DNA, and the amount of DNA detected throughout development is well illustrated in blots of embryos resulting from sperm incubated with 100 µg/ml of linear pRSV-lacZ DNA (Figures 4.5 and 4.6) and

Figure 4.4. Detection of circular pRSV-lacZ DNA in embryos by Southern analysis. DNA was extracted from 1.2 day old embryos resulting from sperm incubated with 100 µg/ml of circular pRSV-lacZ DNA. Lane 1: circular pRSV-lacZ DNA (100 pg); 2: circular pRSV-lacZ DNA (10 pg); 3: circular pRSV-lacZ DNA (1 pg); 4: 5 µg of salmon genomic DNA (the same DNA solution added to the embryo DNA samples to assist in efficient precipitation during the extraction procedure); 5 to 9: embryos resulting from un-shocked sperm (no pulse); 10 to 14: sperm exposed to one 1000 V/cm × 18.6ms pulse; 15-19 sperm exposed to two 1000 V/cm × 27.4 ms pulses. The positions of the nicked open circular (NC), covalently closed circular (CC) and supercoiled (SC) are indicated.

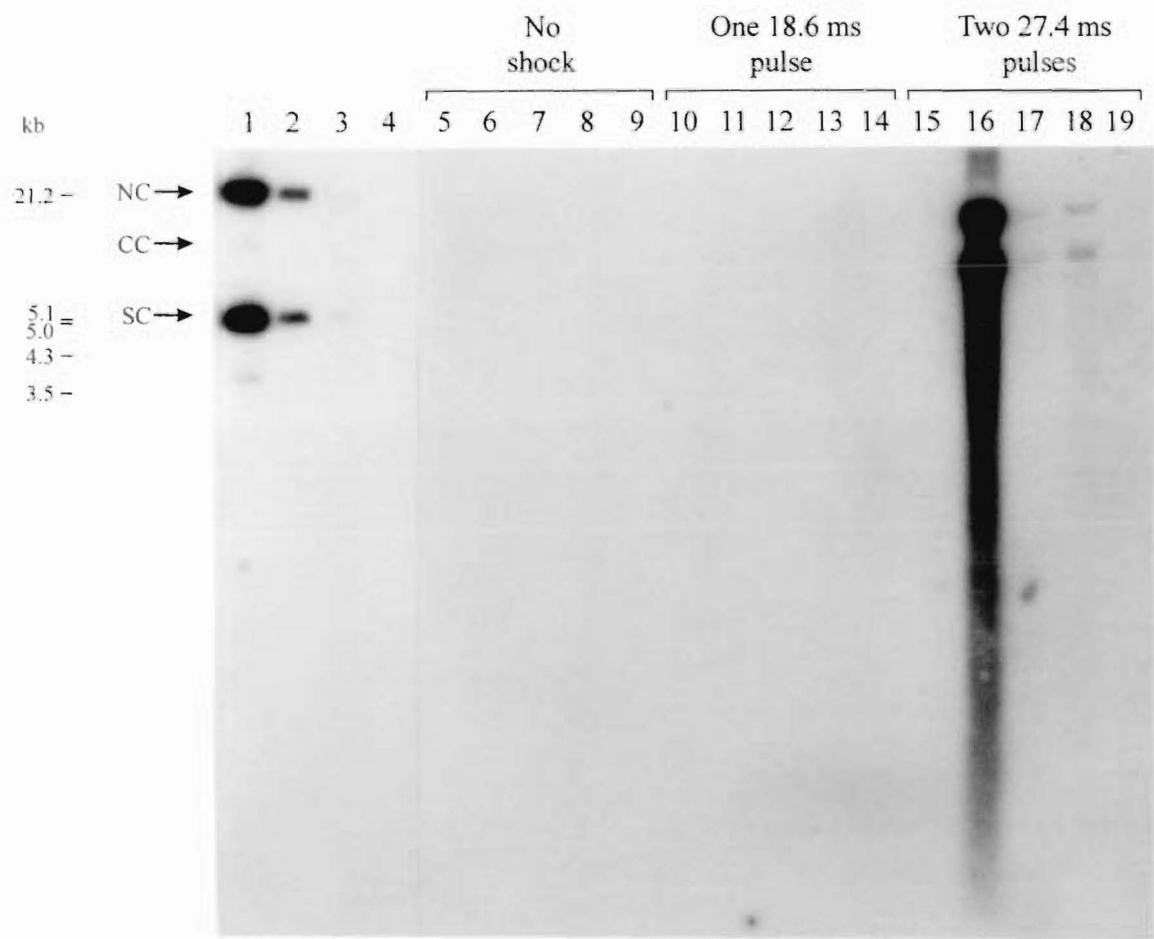


Figure 4.5. Detection of linear pRSV-lacZ DNA in 1.2, 3.3 and 4 day old embryos by Southern analysis. DNA was extracted from embryos resulting from sperm electroporated with two 1000 V/cm \times 27.4 ms pulses in the presence of 100 μ g/ml of linear pRSV-lacZ DNA. Lane 1: linear pRSV-lacZ DNA (100 pg); 2: linear pRSV-lacZ DNA (10 pg); 3: linear pRSV-lacZ DNA (1 pg); 4: 5 μ g of salmon genomic DNA (the same DNA solution added to the embryo DNA samples to assist in efficient precipitation during the extraction procedure); 5 to 9: 1.2 day old embryos; 10 to 14: 3.3 day old embryos; 15-19: 4 day old embryos.

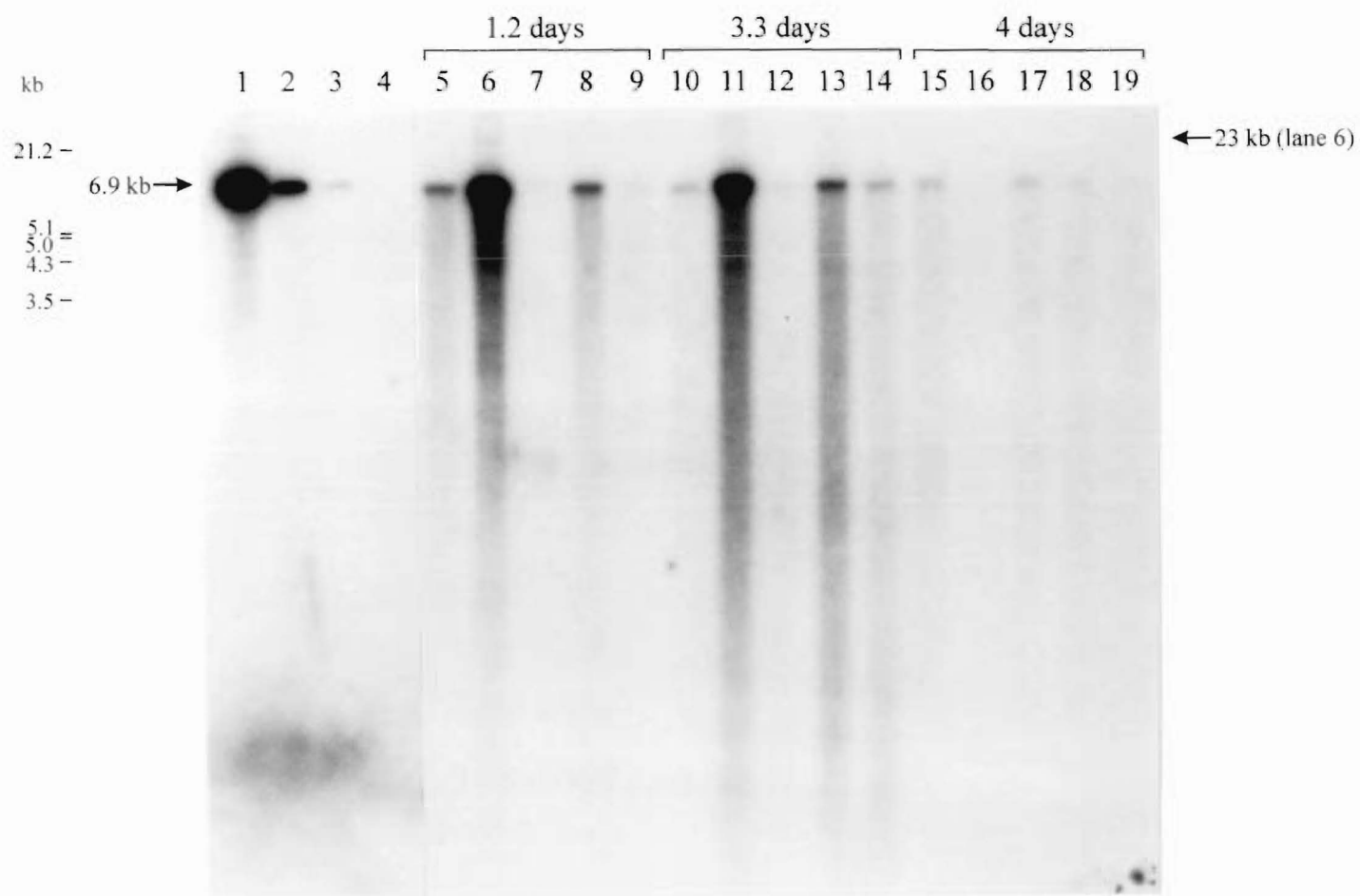
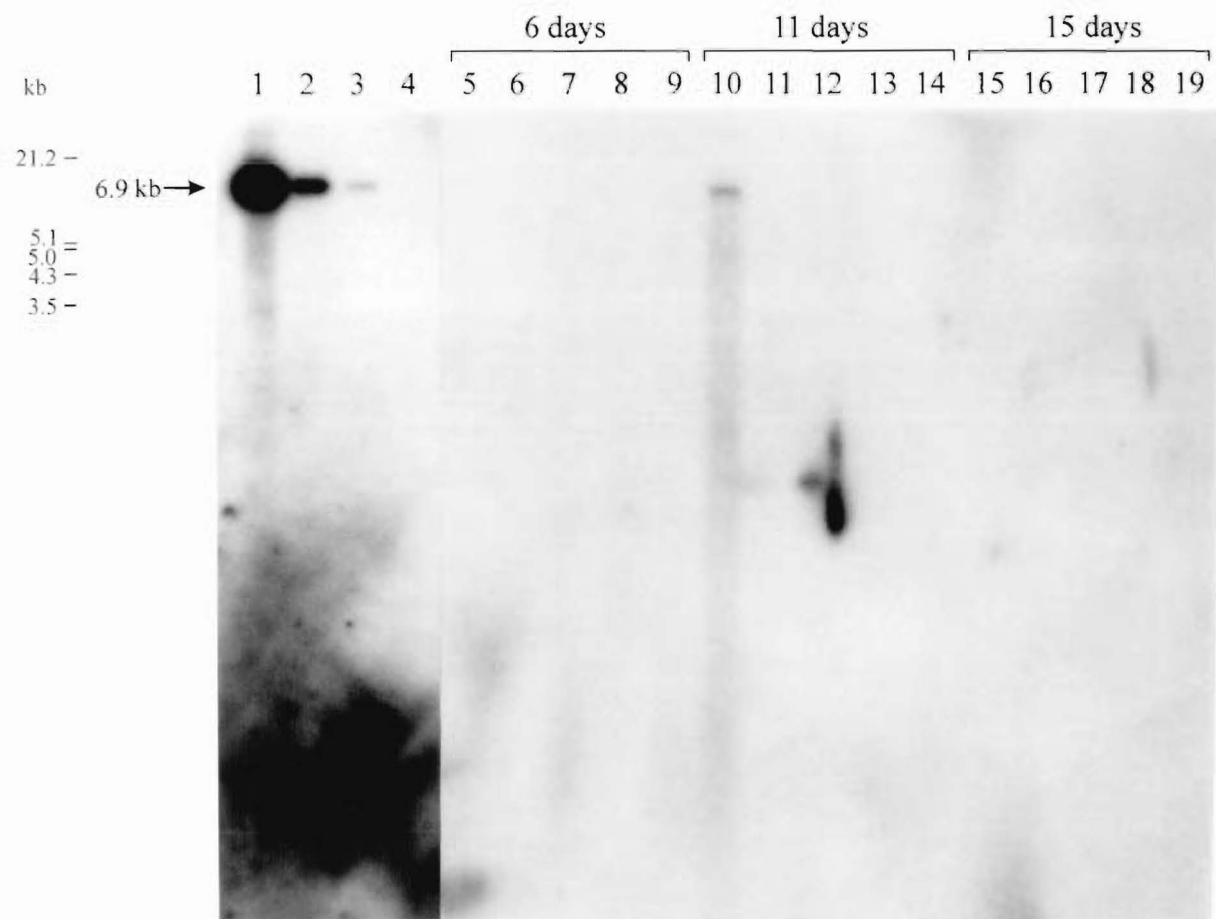


Figure 4.6. Detection of linear pRSV-lacZ DNA in 6, 11 and 15 day old embryos by Southern analysis. DNA was extracted from embryos resulting from sperm electroporated with two $1000 \text{ V/cm} \times 27.4 \text{ ms}$ pulses in the presence of $100 \text{ } \mu\text{g/ml}$ of linear pRSV-lacZ DNA. Lane 1: linear pRSV-lacZ DNA (100 pg); 2: linear pRSV-lacZ DNA (10 pg); 3: linear pRSV-lacZ DNA (1 pg); 4: $5 \text{ } \mu\text{g}$ of salmon genomic DNA (the same DNA solution added to the embryo DNA samples to assist in efficient precipitation during the extraction procedure); 5 to 9: 6 day old embryos; 10 to 14: 11 day old embryos; 15-19: 15 day old embryos.



in Table 4.1. By 6 days post-fertilisation the level of introduced DNA had fallen below the level of detection, although the equivalent of 1×10^5 copies (approximately 1 pg) was present in one 11 day old individual (lane 10, Figure 4.6, 100 $\mu\text{g/ml}$ of linear pRSV-lacZ DNA, 2 pulses of 1000 V/cm \times 27.4 ms). Overall, the pattern of persistence was similar in all treatments irrespective of the electroporation conditions used.

4.3.2 Fate of the DNA: Form of the DNA after transfer into embryos.

Overall, as illustrated in Figures 3.11 (page 74) and 4.5, introduced linear DNA of both pRSV-lacZ and pSV2CAT plasmids remained unmodified, and was detected as a single band with the same mobility as the control plasmid. This was the same in most samples irrespective of the electroporation conditions used. However, there were two exceptions. Firstly, in a linear pSV2CAT treatment (200 $\mu\text{g/ml}$, 2 pulses of 1000 V/cm \times 27.4 ms), 1 band corresponding to a molecular weight of 9.8 kb was observed (lane 16 Figure 3.11, page 74). Secondly, in a linear pRSV-lacZ treatment (100 $\mu\text{g/ml}$, 2 pulses of 1000 V/cm \times 27.4ms) a 23 kb band was detected (lane 6, Figure 4.5).

Several forms of circular DNA were detected in the control lanes on the Southern blots. In the control lanes (lanes 1 to 3, Figures and 3.12 (page 75), 3.14 (page 80) and Figure 4.4) two major forms of both plasmids were obtained; supercoiled DNA (the faster migrating), and nicked open circular DNA. A third fainter band of intermediate mobility, corresponding to approximately 6 kb and 8 kb for pSV2CAT and pRSV-lacZ respectively, probably represents relaxed covalently closed circular DNA. Two other faint bands were observed only in the control lanes and these probably represent different multimeric circular forms of the plasmids. Alternatively the faster migrating bands, 2.7 kb for pSV2CAT and 4 kb for pRSV-lacZ, may represent ejected inserts from the plasmids (actual insert sizes are 2.1 kb for pSV2CAT and 4.9 kb for pRSV-lacZ). The bands of slow mobility, corresponding to approximately 28 kb for pSV2CAT and 86 kb for pRSV-lacZ, may represent multimers of the plasmids.

In the majority of embryo DNA samples resulting from sperm electroporated in the presence of circular DNA (pRSV-lacZ and pSV2CAT), two forms of plasmid DNA were

detected (Figures, 3.12 (page 75), 3.14 (page 80) and Figure 4.4). These were the relaxed covalently closed circular and the relaxed open circular forms. However, the more slowly migrating form of pRSV-lacZ DNA detected in the embryonic samples, migrated slightly faster than the closed relaxed circular form in the control lanes (see Figures 3.14 (page 80) and 4.4). This is probably due to an artefact of the electrophoresis. In three samples from embryos treated with 200 µg/ml circular pSV2CAT DNA and electroporated with 2 pulses 27.4 ms long at 1000 V/cm, extra bands were observed. Firstly, an additional faint band of supercoiled DNA (corresponding to 3284 bp) was detected (lane 17, Figure 3.12 (page 75)). Secondly, two extra bands, corresponding to 20 kb and 35 kb, were faintly observed in another individual (lane 11, Figure 3.12 (page 75)). The size range of the salmon genomic DNA electrophoresed in these gels was approximately 18.5 to 24 kb. Finally, in one other embryo DNA sample a relatively intense band was observed that was smaller (faster migrating) than those observed in similar samples (lane 19, Figure 3.12), and had a relative electrophoretic mobility corresponding to 1540 bp.

When DNA, extracted from embryos into which circular pRSV-lacZ had been transferred (100 µg/ml of plasmid DNA, one pulse of 1000 V/cm × 1.8.6 ms and two pulses of 1000 V/cm × 27.4 ms), was digested with the restriction endonuclease *EcoRI*, the bands observed were of the expected sizes for circular pRSV-lacZ (or head-to-tail concatemers of this plasmid) and were the same as those observed in the control lanes (Figure 4.7).

In addition to the discrete bands observed following Southern analysis of embryonic DNA, there was also evidence that the transferred circular and linear plasmid DNA was being degraded during development. This was demonstrated by the tailing effect observed running down from the bands on the autoradiographs. The degradation is apparent in all the developmental stages examined by Southern analysis, including the 1.2, 3.3, 4, and 11 day post-fertilisation time points and was not related to the sperm electroporation conditions (see Figures 3.11 (page 74), 3.12 (page 75), 3.14 (page 80), and 4.4 to 4.6).

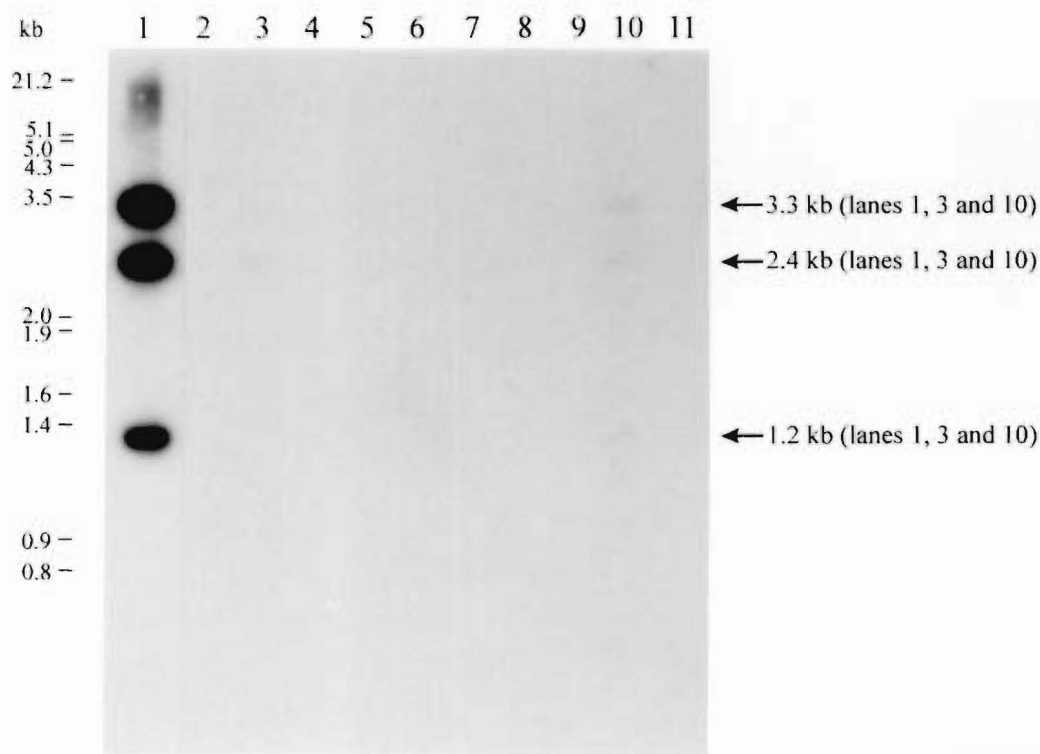


Figure 4.7. Detection of circular pRSVlacZ DNA in 4 day old embryos by Southern analysis. DNA extracted from embryos was digested with *EcoR* I before being analysed by Southern blotting. Lane 1: *EcoR* I digested pRSVlacZ DNA (500 pg); 2 to 6: embryos from the 100 µg/ml, one shock, 1000 V/cm, 18.6 ms treatment; 7 to 11: embryos from the 100 µg/ml, two shock, 1000 V/cm, 27.4 ms treatment

4.3.3 Inverse PCR.

IPCR was carried out on 23 individuals that had been shown to contain pRSV-lacZ DNA by PCR followed by Southern hybridisation analysis. Individuals 15, 26 and 70 days post-fertilisation were tested from the zero, one and two pulse linear DNA treatments. The procedure was applied only to relatively older fish as by these stages DNA existing extrachromosomally is likely to have degraded. This excess DNA will not then soak up primers or be involved in ligation reactions, and thus interfere with the analysis. IPCR carried out on linear plasmid DNA should yield no product, unless re-ligation at the *Apa*I site had occurred due to the formation of concatemers or re-circularisation of the plasmid DNA (see Figure 4.1). Therefore treatments involving linear and not circular plasmid DNA were used.

Only 2 samples yielded products that were visible on the agarose gels following ethidium bromide staining (lanes 5 and 6, Figure 4.8a). These, however, were caused by non-specific binding of the primers as no bands were detected in the same positions upon hybridisation with the plasmid specific probe (Figure 4.9a-c). Several other bands were apparent on the autoradiographs. In five samples, IPCR products were obtained the same size (1277 bp) as predicted for circular plasmid DNA and did not represent integration events (lanes 5, 9 and 11, Figure 4.9a; lane 10, Figure 4.9b; lane 5, Figure 4.9c). These probably represent the re-ligation of the linear DNA in the fish cells to form circular DNA molecules or concatemers. Alternatively such products may be an artefact of the IPCR process caused by ligation of two molecules, at the site of linearisation, instead of small circles forming from single DNA fragments.

In some lanes novel bands were observed following hybridisation (lanes 5 to 12, Figure 4.9a; lanes 4, 5, 6, 10 and 12, Figure 4.9b; lanes 8, 10 and 12, Figure 4.9c), including 3 faint bands with molecular weights higher than expected 1277 bp (lanes 11, Figure 4.9a; lane 10, Figure 4.9b; lane 10, Figure 4.9c). After excision of four slots from replicate agarose gels in the size region of each of the novel sized bands, none could be re-amplified. These products were probably an artefact of the IPCR procedure as they apparently did not contain both (or either) of the primer binding sequences. Given the inability of the fragments to be re-amplified this work was not continued further.

Figure 4.8 Inverse PCR analysis. Following digestion with *Csp6I*, DNA samples were ligated and used as a template for PCR with the primers ISV1 and ISV2 (except for (a) lane 3 where the primers IPRL1 and IPRL2 were used). All the salmon screened by IPCR originated from sperm electroporated with linear pRSV-lacZ DNA.

(a) Lane 1: 1277 bp IPCR control, circular pRSV-lacZ DNA; 2: lambda DNA digested with *EcoRI* and *HindIII* (fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 3: 416 bp inverse PCR control product amplified from the chinook salmon prolactin gene with the primers IPRL1 and IPRL2; 4: 15 day old negative control embryo; 5 to 9: 15 day old embryos from the 100 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment; 10: 26 day old negative control embryo; 11 and 12: 26 day old embryos from the un-shocked 100 µg/ml treatment.

(b) Lane 1: 1277 bp IPCR control, circular pRSV-lacZ DNA; 2: lambda DNA digested with *EcoRI* and *HindIII* (fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 3: water control reaction; 4 and 5: 26 day old embryos from the 100 µg/ml, 1 pulse, 1000 V/cm × 18.6 ms treatment; 6 and 7: 26 day old embryos from the 100 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment; 8: 70 day old negative control fry; 9: 70 day old fry from the 200 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment; 10 and 11: 70 day old fry from the 100 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment; 12: 70 day old fry from the 20 µg/ml, 1 pulse, 1000 V/cm × 18.6 ms treatment.

(c) Lane 1: 1277 bp IPCR control, circular pRSV-lacZ DNA; 2: lambda DNA digested with *EcoRI* and *HindIII* (fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 3: blank lane; 4 to 9: 70 day old fry from the 20 µg/ml, 1 pulse, 1000 V/cm × 18.6 ms treatment; 10 to 12: 70 day old fry from the 20 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment.

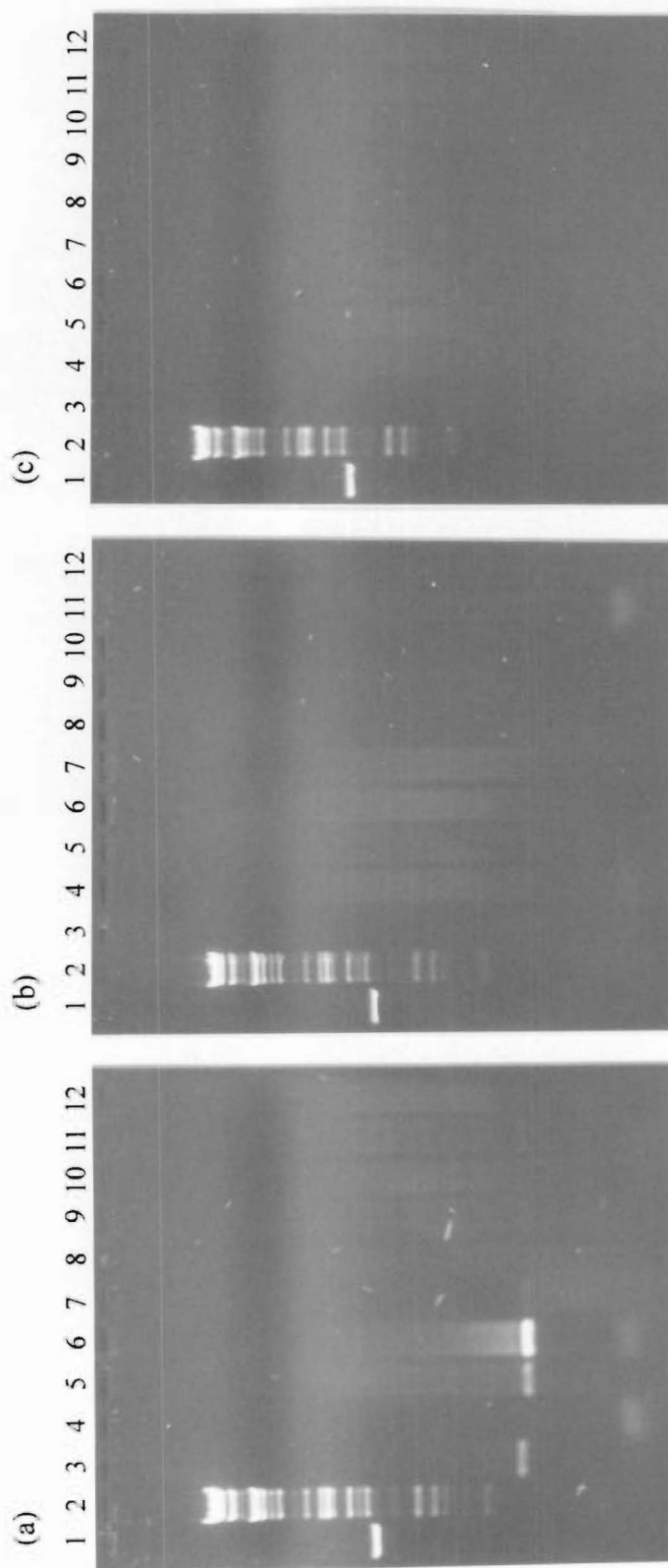
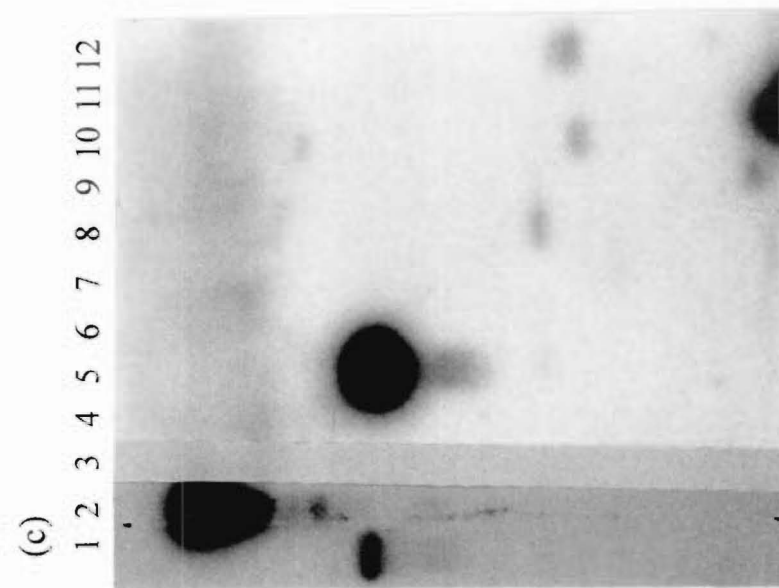
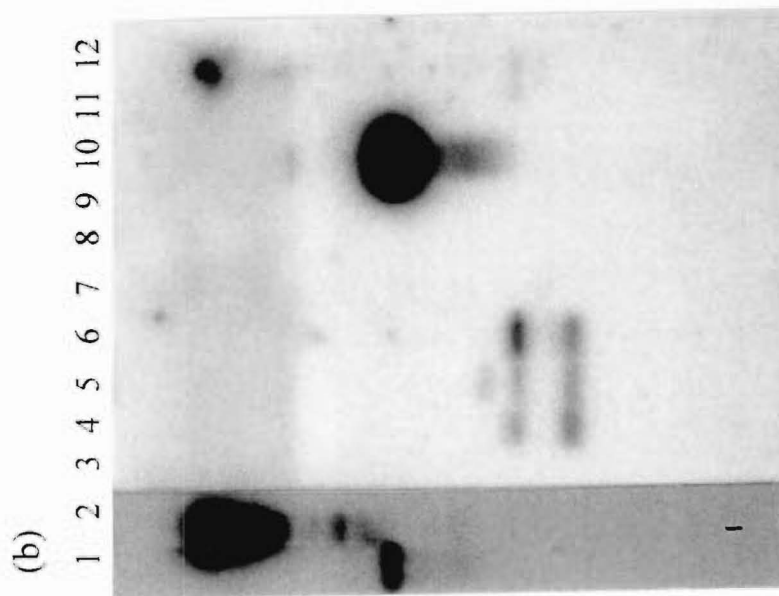
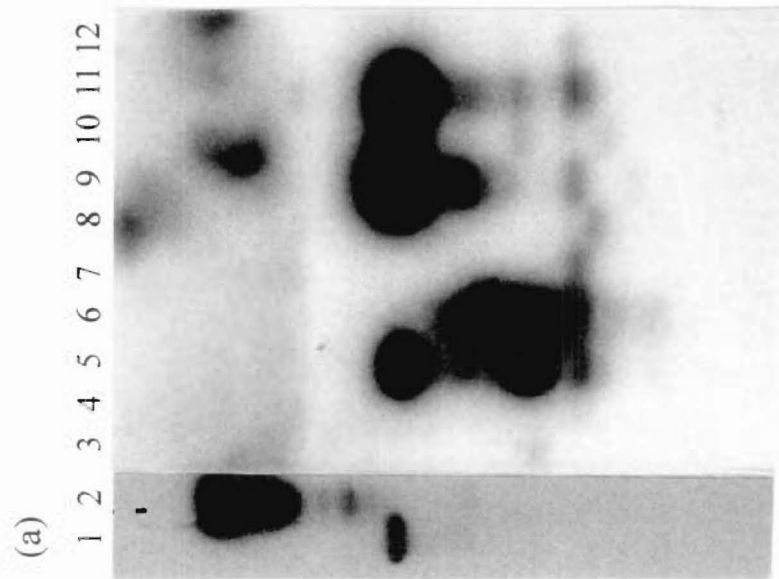


Figure 4.9 Southern analysis of inverse PCR products. DNA from the gels presented in figure 4.8 were transferred to nylon membrane and hybridised with a probe specific to pRSV-lacZ DNA but not to the ISV1 and ISV2 primer bindings sequences (see figure 4.1). After hybridisation the membranes were exposed to X-ray film for 9 days, except for lanes 1 and 2 (a, b and c) which were exposed for 1 hour and 15 hours respectively.

(a) Lane 1: 1277 bp IPCR control, circular pRSV-lacZ DNA; 2: lambda DNA digested with *EcoRI* and *HindIII* (fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 3: 416 bp inverse PCR control product amplified from the chinook salmon prolactin gene with the primers IPRL1 and IPRL2; 4: 15 day old negative control embryo; 5 to 9: 15 day old embryos from the 100 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment; 10: 26 day old negative control embryo; 11 and 12: 26 day old embryos from the un-shocked 100 µg/ml treatment.

(b) Lane 1: 1277 bp IPCR control, circular pRSV-lacZ DNA; 2: lambda DNA digested with *EcoRI* and *HindIII* (fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 3: water control reaction; 4 and 5: 26 day old embryos from the 100 µg/ml, 1 pulse, 1000 V/cm × 18.6 ms treatment; 6 and 7: 26 day old embryos from the 100 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment; 8: 70 day old negative control fry; 9: 70 day old fry from the 200 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment; 10 and 11: 70 day old fry from the 100 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment; 12: 70 day old fry from the 20 µg/ml, 1 pulse, 1000 V/cm × 18.6 ms treatment.

(c) Lane 1: 1277 bp IPCR control, circular pRSV-lacZ DNA; 2: lambda DNA digested with *EcoRI* and *HindIII* (fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 3: blank lane; 4 to 9: 70 day old fry from the 20 µg/ml, 1 pulse, 1000 V/cm × 18.6 ms treatment; 10 to 12: 70 day old fry from the 20 µg/ml, 2 pulse, 1000 V/cm × 27.4 ms treatment.



4.3.4 β -gal expression.

4.3.4.1 Histochemical assay using X-gal.

Individuals of several embryonic stages from treatments that had previously been shown to produce a high proportion of embryos containing pRSV-lacZ DNA, were tested for β -gal expression. A total of ninety-five embryos, from 3 treatments and 7 different age groups (3.3 to 43 days old), were dissected and tested in this way (see Table 4.2). For stages younger than 11 days old, embryos were dissected and separated from the yolk and oil by centrifugation. When whole embryos were fixed without first being dissected the fixed yolk became brown and opaque and obscured the embryo. No blue staining, indicating β -gal expression, was evident in embryos 3.3 to 15 days old. In fish 26, (stage 22) and 43 (stage 24) days post-fertilisation, blue staining was observed. However, this was endogenous background expression and/or of bacterial origin and was also found in normal control fish, with blue staining being observed in the gut and spine areas (see Figure 4.10).

4.3.4.2 Spectrophotometric assay using ONPG.

The ONPG substrate was used to assay expression because no expression above background was observed with X-gal staining. Additionally, recovery of fixed embryonic cells of the earliest developmental stages for the X-gal staining procedure proved to be problematic. It was important to assay expression in these developmental stages as they were more likely to contain detectable amounts of β -gal due to the higher levels of pRSV-lacZ DNA present.

Table 4.3 shows the number of samples, the treatments and ages of the embryos screened by the ONPG method. Two individuals had clearly elevated readings (marked by an * in Table 4.3 and Figure 4.11a, b and c): (1) in a morula stage embryo (1.2 days old) in the 2×27.4 ms, 100 μ g/ml circular DNA treatment an OD₄₂₀ of 0.084 resulted (Figure 4.11a) and (2) an OD₄₂₀ of 0.049 was observed in an embryo 3.3 days post-fertilisation in the 1×18.6 ms, 200 μ g/ml linear DNA treatment (Figure 4.11b). After allowing for the maximum background readings in control embryos, 0.026, these OD₄₂₀ readings correspond to the equivalent of approximately 2×10^{-3} and 7.4×10^{-4} units of β -gal respectively. Although readings above background levels resulted for some of the other samples, they were only marginally elevated, with OD₄₂₀ readings of 0.027 to 0.030 (Figure 4.11a-c). Additionally, unlike the two clearly positive samples, no others exhibited the yellow end point characteristic of β -gal activity.

Table 4.2. Electroporation conditions and ages of embryos screened for β -galactosidase expression histochemically using X-gal, after the transfer of circular (C) pRSV-lacZ. The electroporation conditions were no electroporation (0), 1 pulse 18.6 ms long of 1000 V/cm (1) and 2 pulses 27.4 ms long of 1000 V/cm (2).

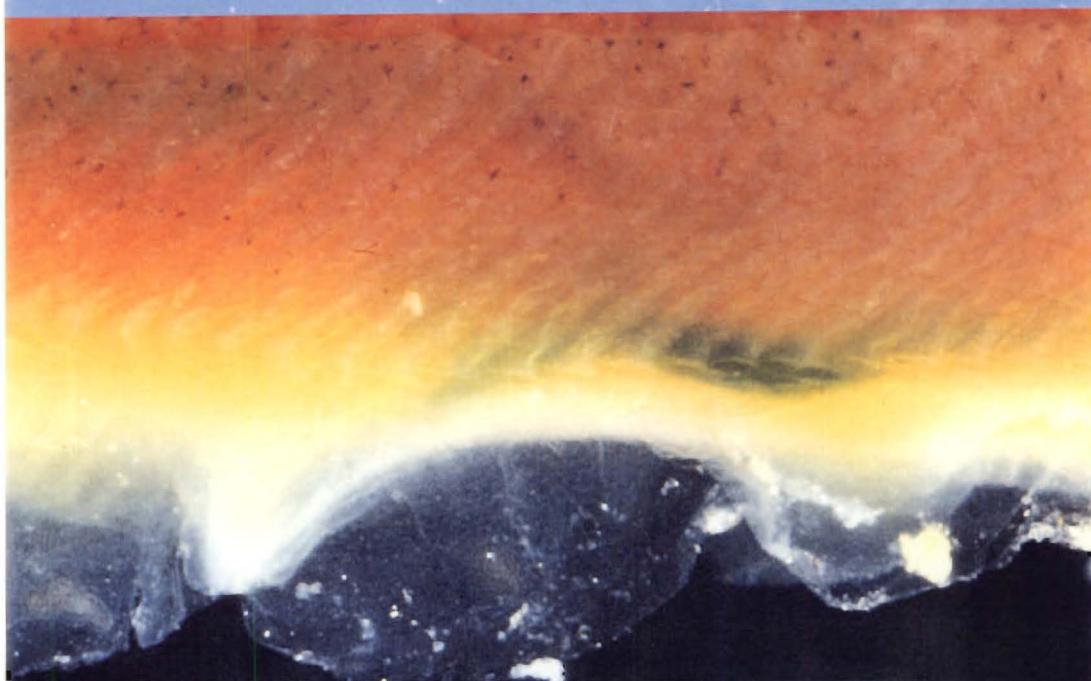
DNA form	DNA concentration (μ g/ml)	Electroporation condition	Number of days post fertilisation						
			3.3	4	6	11	15	26	43
			Number of embryos screened						
-	-	0	5	5	5	5	5	5	5
C	100	1	5	3	5	3	5	4	4
C	100	2	5	4	4	5	5	4	4

Figure 4.10. Example of the background β -D-galactosidase activity detected in control embryos by histochemical staining using X-gal. (a) A 43 day old fish (just hatched) is shown (actual length 11 mm), with close ups of, (b) the anal area and (c) the tail region. Blue staining, indicating the presence of β -D-galactosidase, can be observed in the gut and spine areas. The yolk sac has been removed.

(a)



(b)



(c)

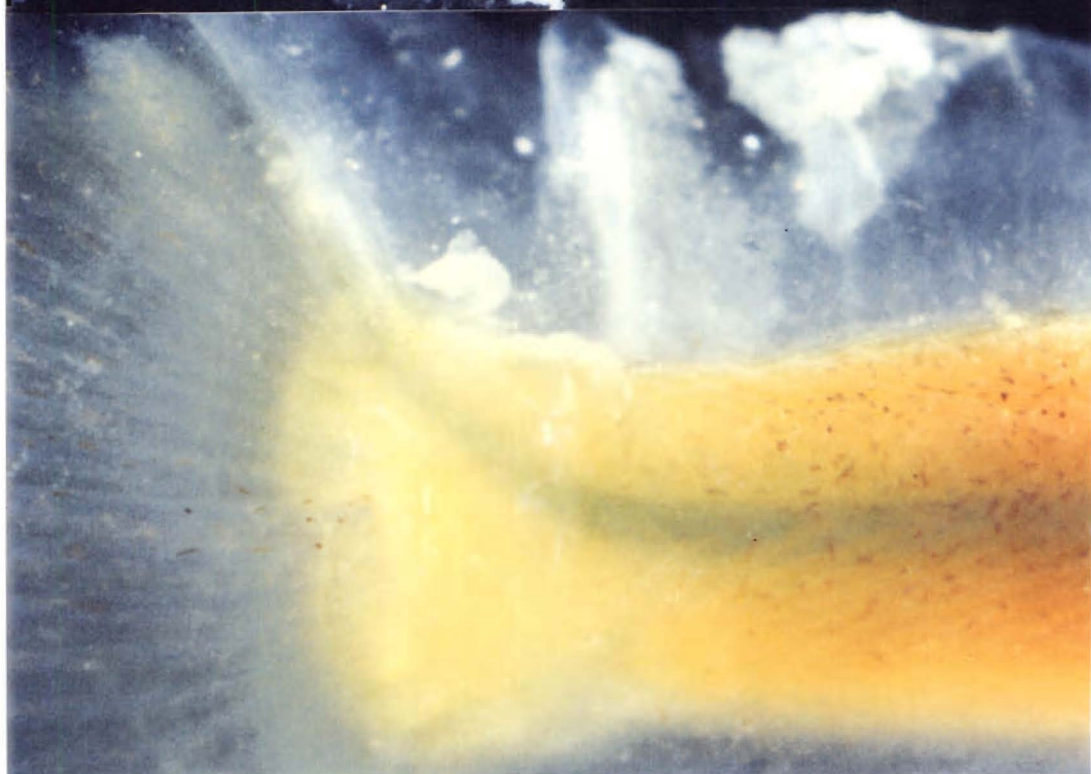
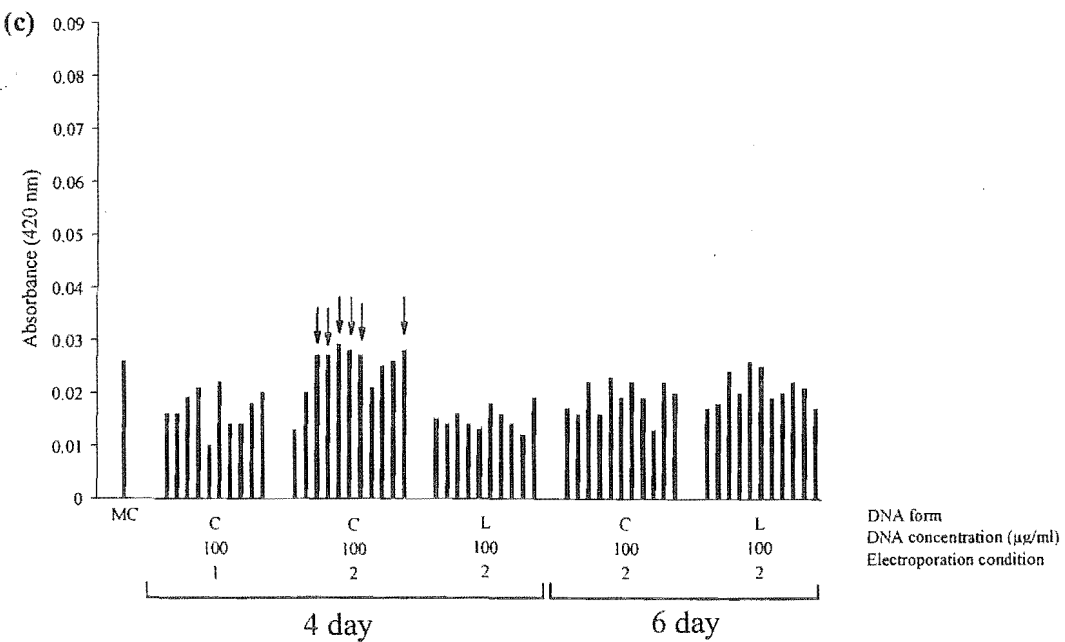
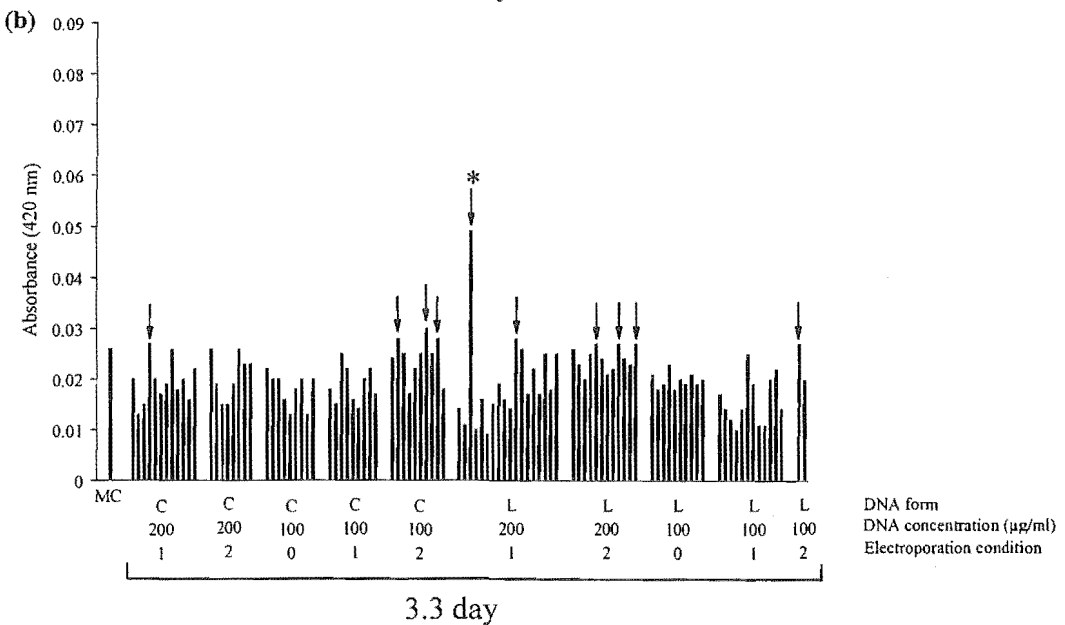
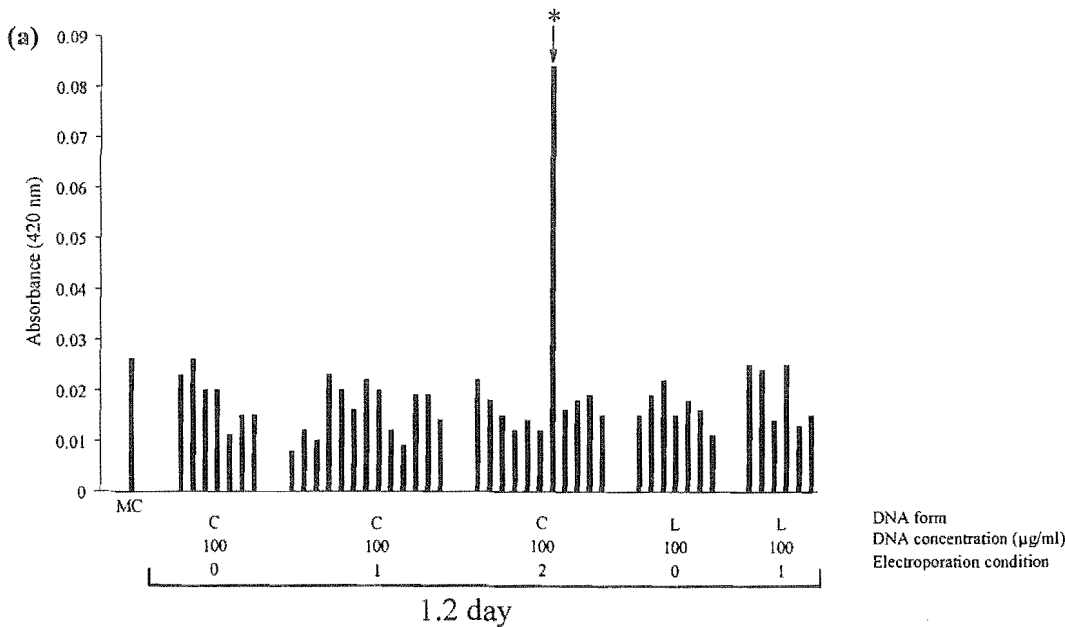


Table 4.3. Electroporation conditions and ages of the embryos screened for β -galactosidase expression using ONPG after the transfer of circular (C) and linear (L) pRSV-lacZ. The electroporation conditions were no electroporation (0), 1 pulse 18.6 ms long of 1000 V/cm (1) and 2 pulses 27.4 ms long of 1000 V/cm (2). The treatments in which expression was detected are indicated by *. NS = not screened.

DNA form	DNA concentration (µg/ml)	Electroporation condition	Number of days post fertilisation			
			1.2	3.3	4	6
			Number of embryos screened			
-	-	-	20	24	13	6
C	200	1	NS	12	NS	NS
C	200	2	NS	9	NS	NS
C	100	0	7	9	NS	NS
C	100	1	10	9	10	NS
C	100	2	*11	10	11	11
L	200	1	NS	*18	NS	NS
L	200	2	NS	12	NS	NS
L	100	0	7	10	NS	NS
L	100	1	6	12	NS	NS
L	100	2	NS	2	10	11

Figure 4.11a, b and c. β -gal expression in embryos detected by ONPG analysis after the transfer of pRSV-lacZ by electroporated salmon sperm. Both circular (C) and linear (L) treatments were screened. The electroporation conditions were no electroporation (0), 1 pulse 18.6 ms long of 1000 V/cm (1) and 2 pulses 27.4 ms long of 1000 V/cm (2). DNA concentrations of 100 or 200 μ g/ml were used. The age of the embryos (1.2, 3.3, 4 or 6 days post-fertilisation) is indicated. The maximum level of absorbance at 420 nm obtained in control embryos is indicated (MC). Individuals with absorbances above the maximum control level are indicated by an arrow. The two individuals with the most elevated levels, and the only samples for which a yellow end point was observed, are also indicated (*).



4.3.5 CAT expression.

Individuals of several embryonic stages from treatments that had previously been shown to produce a high proportion of embryos containing pSV2CAT DNA, were tested for CAT expression. Table 4.4 shows the number of embryos, the treatments and ages screened for CAT activity using ^{14}C -chloramphenicol and TLC. Positive controls, when 0.1 units of purified CAT were added to a normal negative embryo extract, showed activity in the form of dark images upon autoradiography corresponding to 3-acetate chloramphenicol and 1-acetate chloramphenicol (lanes 1 and 4, Figure 4.12a; lane 1, Figure 4.12b). In the negative controls (normal embryos) no evidence of CAT activity was detected (lane 2, Figure 4.12a.). No clear evidence of expression was seen in any of the extracts from experimental embryos. Some faint images were obtained upon autoradiography of TLC plates (for example, lane 9, Figure 4.12a and lanes 6, 8 and 9, Figure 4.12b), many of which did not correspond to the mobilities of the acetylated forms of chloramphenicol observed in the control lanes. In one embryo faint bands corresponding to the 1 and 3-acetate chloramphenicol were observed (lane 2, Figure 4.12b). This occurred in an embryo originating from sperm electroporated with two pulses of $1000\text{ V/cm} \times 27.4\text{ ms}$ in the presence of $200\text{ }\mu\text{g/ml}$ of circular pSV2CAT DNA.

Table 4.4. Electroporation conditions and ages of the embryos screened for chloramphenicol acetyltransferase expression. ^{14}C -chloramphenicol and thin layer chromatography were used to test for expression of transferred circular (C) and linear (L) pSV2CAT. The electroporation conditions were no electroporation (0), 1 pulse 18.6 ms long of 1000 V/cm (1) and 2 pulses 27.4 ms long of 1000 V/cm (2). NS = not screened.

DNA form	DNA concentration (µg/ml)	Electroporation condition	Days since fertilisation	
			1.2	3.3
			Number of embryos screened	
-	-	-	5	10
C	200	1	5	NS
C	200	2	5	5
L	200	2	NS	5

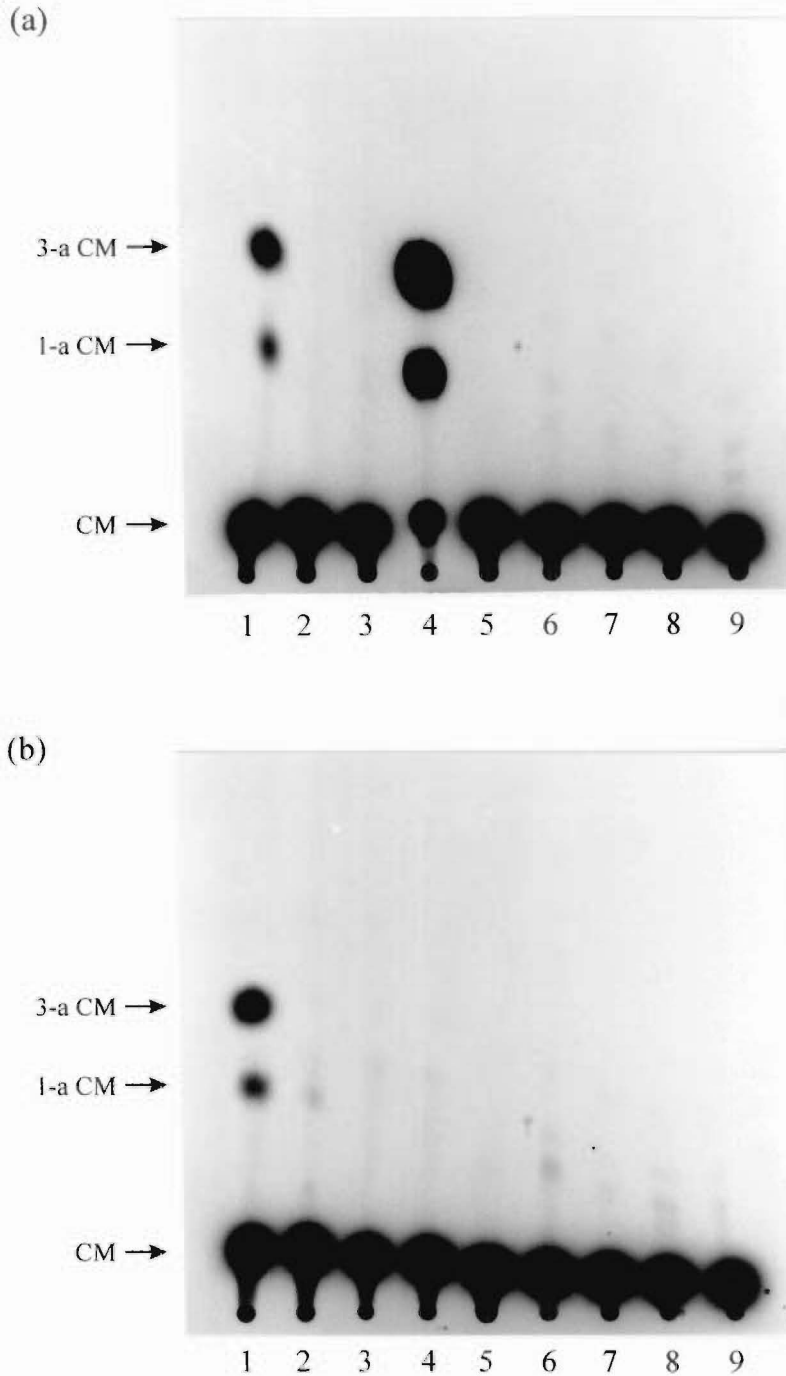


Figure 4.12. Screening of chloramphenicol acetyltransferase (CAT) expression by thin layer chromatography. (a). Lane 1: 0.1 unit of CAT; 2: negative control, no extract or enzyme added; 3: negative control embryo; 4: extract from a negative control embryo to which 0.1 units of CAT was added prior to the assay; 5 to 9: 1.2 day old embryos from the 200 $\mu\text{g}/\text{ml}$ circular DNA, 1 pulse, 1000 V/cm, 18.6 ms treatment. (b). Lane 1: 0.1 unit of CAT; 2 to 6: 1.2 day old embryos from the 200 $\mu\text{g}/\text{ml}$ circular DNA, 2 pulse, 1000 V/cm, 27.4 ms treatment; 7 to 9: 3.3 day old embryos from the 200 $\mu\text{g}/\text{ml}$ circular DNA, 2 pulse, 1000 V/cm 27.4 ms treatment. The positions of chloramphenicol (CM) 1-acetate chloramphenicol (1-a CM) and 3-acetate chloramphenicol (3-a CM) in lane 1 are indicated.

4.4 Discussion.

In Chapter 3 up to 85% of fry were found to contain introduced plasmid DNA following fertilisation with electroporated sperm. However, the levels of introduced DNA detected in the fry were low, as they could not be detected by Southern analysis alone. As low levels had been observed in fry it was surprising to find such high levels of introduced DNA in early salmon embryos (greater than 100 pg in some cases). These levels are comparable to the levels detected after DNA was microinjected into medaka (Chong and Vielkind, 1989; Winkler *et al.*, 1991) and zebrafish (Stuart *et al.*, 1988). The observed DNA levels also compare favourably with the amounts delivered into embryos when microinjection is used to create transgenic salmonids. The microinjection of approximately 10 pg of plasmid DNA into Atlantic salmon eggs prior to first cleavage is sufficient to obtain a gene transfer efficiency of up to 6% (Fletcher *et al.*, 1988).

In studies examining the fate of DNA microinjected into fish eggs, the introduced DNA was observed to be amplified in early development (from the 32-64 cell stage) to levels up to twelve times that originally injected, with the levels peaking in the gastrula/neurula stage (Chong and Vielkind, 1989; Winkler *et al.*, 1991; Kozlov *et al.*, 1988; Stuart *et al.*, 1988; Zhu, 1992; Volckaert *et al.*, 1994). In this study, when similar developmental stages were analysed, comparable amounts of DNA were detected after transfer by electroporated salmon sperm. However, no direct evidence of amplification of plasmid DNA was observed as levels were already high in the earliest stage examined (morula stage, 1.2 days old). Therefore the level of DNA was only seen to decrease as the age of the salmon embryos increased.

It is most likely that the relatively high levels of plasmid DNA found in early salmon embryos in this study resulted from replication of the DNA following transfer by electroporated sperm. As previously discussed in Chapter 2, when salmon sperm are electroporated in the presence of 100 µg/ml of plasmid (pRSV-lacZ) DNA, a maximum of 1.5 fg (200 copies) of the DNA appears to be strongly associated on average with each sperm (including DNase sensitive DNA). The amount of DNA that is available to be transferred into the embryo upon fertilisation may be greater than this if the viable sperm

preferentially take up more DNA relative to those lysed by the electroporation. However, even if this were true, the amount of DNA associated with a single sperm would still be in the order of tens of femtograms (approximately 1000 copies). Therefore this can not account for the high levels of DNA detected in salmon embryos (greater than 100 pg, 10^7 copies). Additionally, the level of plasmid DNA observed in salmon embryos is over thirty times the amount of genomic DNA that sperm normally carry.

Undoubtedly significantly less DNA is transferred into embryos by individual electroporated sperm than in egg microinjection procedures (10 fg compared to 10 to 100 pg). The discovery of comparative amounts of DNA in later stages may be explained by the sperm delivering this DNA more precisely, thus localising it to the site of the developing embryo or pronuclei fusion, providing that this is followed by replication of the DNA. If this is the case then less DNA may be required to be transferred by sperm. A large proportion of DNA microinjected into fish eggs is undoubtedly degraded, lost due to diffusion into the egg, and/or not deposited near the site of blastodisc formation, and thus not incorporated into the embryo.

Any amplification of the DNA transferred by electroporated salmon sperm must have occurred earlier in development than the stages sampled, and at an earlier developmental stage than in the warm-water species studied (medaka and zebrafish), where the peak DNA level was seen in the late blastula to neurula stage (eg. Chong and Vielkind, 1989). The earliest stage of salmon development examined was the morula stage at 1.2 days, when the embryo consists of hundreds of cells. Salmon have a much slower development than these warm water species, for example, at about 24 hours post-fertilisation medaka are at the late gastrula stage and salmon the morula stage, and salmon embryos do not reach gastrulation until approximately 90 hours (3.75 days) post-fertilisation (the precise timing depends on temperature and species). It is hypothesised that the amplification of exogenous DNA is linked to the stages where the most rapid cell division is occurring between the blastula to neurula stages (Gong and Hew, 1995). However, the amplification of DNA introduced into salmon by electroporated sperm appears to have occurred at a similar time after fertilisation, but at an earlier

developmental stage than the more quickly developing warm water species. Therefore, the results in this chapter suggest that the amplification schedule may be more time dependent than stage specific.

The actual mechanism of replication of introduced DNA and the reason for its timing is not known. Kozlov *et al.* (1988) on observing DNA amplification peaking in loach gastrula, suggested that the amplification of DNA during early embryogenesis is due to cytoplasmic polymerases, and the decrease in introduced DNA levels occurs when these enzymes migrate into the forming nuclei. Such spatial distribution of polymerases has been observed in sea urchins (Fransler and Loeb, 1969). Other investigators have suggested that maternal enzymes are responsible for conformational changes and replication of introduced DNA in the very early *Xenopus* embryo (Zierler *et al.*, 1985; Marini *et al.*, 1988). Two other possible explanations for the replication of introduced DNA were put forward by Winkler *et al.* (1991); following cytoplasmic injection of the DNA significant amounts could end up in the nucleus and undergo replication there. Alternatively, replication may occur in nucleus like particles such as those observed to form around phage DNA in *Xenopus* embryos (Forbes *et al.*, 1983). Whatever the mechanism of replication of the introduced plasmid DNA, it is possible that the controls over DNA replication in the early embryo are not as specific and become more so as the embryo develops (Fletcher and Davies, 1991). This could be crucial for the replication of such introduced DNA because of their lack of eukaryotic replication initiation sequences.

Another notable difference between the behaviour of DNA introduced by electroporated salmon sperm was that, unlike microinjected DNA, it was not transformed rapidly into concatemers or high molecular weight DNA. In embryos into which linear DNA was transferred only the linear form was detected (with two possible exceptions). Although it is possible that the bands observed upon Southern analysis represent a concatemer with the same mobility as the linear molecules, this is unlikely. Similar to linear DNA, circular DNA transferred by electroporated sperm was not observed to form extensive concatenated forms. The two forms of circular DNA detected (Figures, 3.12 (page 75), 3.14 (pages 80) and 4.4) most likely represent the relaxed nicked and covalently

closed circular forms of DNA. Therefore it appears that the supercoiled DNA originally introduced had been converted into these forms. When this DNA was digested with *EcoRI* the banding pattern indicated the presence of only circular DNA (or head-to-head concatemers). Some faint bands of novel mobilities were seen in four individuals, resulting from sperm electroporated with two pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$. These may represent multimeric forms of the introduced plasmid DNA and provide some evidence of the formation of low amounts of concatemers. However, by far the majority of sperm transferred plasmid DNA remained unmodified in salmon embryos.

In studies that have examined the behaviour of introduced DNA in *Xenopus* embryos, the replication of plasmid DNA has been observed following the introduction of circular and linear DNA (Rusconi and Schafner, 1981; Marini *et al.*, 1988; Endean and Smithies, 1989; Asano and Shiokawa, 1993). Circular DNA was observed to be replicated in the circular form as well as following its transformation into high molecular weight concatemers. However, only the concatenated form of linear DNA was observed to be amplified and no increase in the amount of linear DNA molecules occurred. The replication of introduced linear DNA in sea urchin embryos followed the same pattern (McMahon *et al.*, 1985). It appears transformation of linear plasmid DNA into higher molecular weight concatemers is a prerequisite for replication. The reason for this may be because larger DNA molecules are more efficiently replicated (Endean and Smithies, 1989). Size has also been shown to play a positive role in the stability and replication of artificial chromosomes in yeast (Murray *et al.*, 1986). In contrast to these studies, the replication of circular and linear plasmid DNA transferred by electroporated salmon sperm seems to have occurred without it first being transformed into high molecular weight concatemers.

The plasmid DNA transferred into fish by microinjection and subsequently concatenated is found in head-to-tail tandem arrays, or in random orientations as head-to-head, or tail-to-tail arrays (for example, Stuart *et al.*, 1988; Chong and Vielkind, 1989; Iyengar and Maclean, 1995). Head-to-head patterns may be the result of homologous recombination and concatemers consisting of random arrangements indicate

the role of ligases in the modification of the DNA. Other investigators have found evidence of rearrangements of the introduced DNA, pointing to the modification by restriction endonucleases (Kozlov *et al.*, 1988). In comparison DNA introduced by electroporated sperm does not appear to have come into contact with such enzymes. The unmodified state of the linear DNA in particular indicates that no ligation has taken place.

Due to a lack of information on the behaviour of introduced DNA during salmonid embryogenesis the differences observed between DNA microinjected into medaka and zebrafish eggs, and DNA transferred into salmon by electroporated sperm, may not be solely attributed to the transfer technique, but could be species specific. Iyengar and Maclean (1995) demonstrated that concatemerisation of introduced linear and circular DNA does occur in rainbow trout following microinjection. However, no observations of the behaviour of the introduced DNA during development were carried out. The behaviour of cytoplasmic polymerases may differ in salmon, and DNA replication may be due to maternal stores of enzymes laid down in the eggs and occur at the very early stages of development. However, this would not explain how the introduced linear DNA was amplified without first being concatenated, although it is possible that the DNA is replicated by a different process after transfer by sperm compared to the amplification of DNA observed after microinjection.

Overall, no conclusive evidence of genomic integration of the DNA transferred by electroporated salmon sperm was found. However, the appearance in one individual of a band with the same mobility as high molecular weight genomic DNA (lane 11, Figure 3.12 (page 75)) may represent an integration event. If this is occurring then it must be at a very low frequency as such bands were rare and faint, and no DNA was detected at appreciable levels in later developmental stages. When fry DNA samples, shown to be positive by PCR analysis, were screened by Southern analysis no positive signals were obtained (Chapter 3). This does not however, rule out the possibility that the DNA detected in fry samples is integrated, but the level must be low and the fish highly mosaic.

IPCR analysis of the later embryonic stages and fry DNA samples did not yield any conclusive evidence of integration of the introduced DNA. When IPCR products were analysed by Southern hybridisation, bands of novel size were detected in several individuals. However, as most of these were of the same size between individuals, they most likely represent artefacts of the IPCR process rather than separate integration events. These bands may be restriction fragments of the original introduced pRSV-lacZ DNA (if present at high levels extrachromosomally), or fragments arising from non-specific priming during the PCR, that contain sequence that binds to the probe but not to one or both of the primers. None of the novel sized bands could be re-amplified. Therefore, as no further analysis, such as sequencing, was carried out on these fragments, the presence of amplified junction sequence following integration was not verified. Putative evidence of integration following transfer into fish by electroporated sperm has been obtained by other researchers. When the opAFPGHc construct was introduced into loach it was found to persist in 6 month old individuals and novel restriction fragments were obtained upon Southern analysis (Tsai *et al.*, 1995).

pRSV-lacZ expression analysis demonstrated that the DNA transferred by electroporated salmon sperm can be expressed, albeit at a very low frequency. The 2 fish testing positive for β -gal expression were detected in a double shock, 1000 V/cm \times 27.4 ms (with 100 μ g/ml of circular DNA) treatment and in a high DNA concentration (200 μ g/ml) treatment with linear DNA and one shock of 1000 V/cm \times 18.6 ms, in the earliest developmental stages (1.2 and 3.3 days old respectively). The ages and electroporation conditions correspond to treatments where high levels of DNA were detected by Southern analysis. In contrast, only very weak evidence of pSV2CAT expression in one salmon embryo was obtained. This occurred in a 1.2 day old embryo originating from sperm electroporated with two pulses of 1000 V/cm \times 27.4 ms in the presence of 200 μ g/ml of circular pSV2CAT DNA.

The results of the expression analysis are significant as they demonstrate that pSV2CAT and pRSV-lacZ DNA transferred by electroporated sperm is capable of expression in salmon embryos. Expression of DNA introduced by electroporated sperm

has also been demonstrated in African catfish when the product of pHSVtk/neo construct was detected (Müller *et al.*, 1992). Additionally, when the opAFPGHc construct was introduced into loach by electroporated sperm evidence of expression of the introduced DNA was obtained as the growth rate was increased in the fish groups resulting from treated sperm (Tsai *et al.*, 1995).

4.4.1 Summary.

The results described in this chapter have demonstrated that electroporated salmon sperm can transfer DNA into a high proportion of embryos, that the levels of DNA are high during early development, and that the introduced DNA can be expressed. This is an important finding as large amounts of exogenous DNA can easily be transferred on mass into salmon using this technique. However, the amount of DNA present decreases as the fish develop and levels in the fry are below the level of detection by Southern analysis. No convincing evidence of integration of the introduced DNA was obtained.

The study of the fate of the DNA transferred by electroporated salmon sperm has shown that there are clear differences between the plasmid DNA transferred by this method and that introduced into other fish species by egg microinjection. The sperm transferred DNA remains almost exclusively unmodified and there is little evidence of replication during development. It is possible that the DNA transferred by the two techniques experiences a different enzymatic environment and may be located in different compartments of the embryonic cells.

Chapter Five.

Growth Hormone gene transfer.

5.1 Introduction.

To further evaluate the use of sperm electroporation for gene transfer in salmon a gene construct known to enhance the growth of Pacific salmon was used. As the evaluation could be done on the basis of phenotype (weight), as well as DNA screening, this enabled the screening for transferred DNA in much larger numbers of fish than in previous experiments. Although transfer into salmon embryos using electroporated sperm has been demonstrated in the previous chapters, only low levels of introduced DNA were detected in fry stage salmon. However, due to the number of variables tested and the screening methods used, only relatively small numbers were screened, and transgenic individuals may have been overlooked. If sperm electroporation results in only a small percentage of true transgenics, then the screening of a large number of individuals increases the likelihood that these will be detected. In addition, expression of the introduced gene is essential if sperm electroporation is to be employed routinely. Expression studies using genes such as *CAT* or *lacZ* are expensive, time consuming and technically difficult. Therefore as a test of the ability of genes to be expressed following transfer by electroporated sperm, a construct which produces an obvious phenotypic effect enables expression to be tested effectively.

There have been several reports of transgenic fish with increased growth rates mediated by the transfer of growth hormone genes. The transfer of a mouse metallothionein promoter-human GH gene construct resulted in loach and carp 3 to 4 times larger than normal (Zhu, 1992). Transgenic carp with less elevated growth rates (20 to 60%) were also obtained by the transfer of a RSV long terminal repeat-rainbow trout GH cDNA gene construct (Zhang *et al.*, 1990; Chen *et al.*, 1993). A similar moderate growth enhancement of medaka was also observed upon the introduction of the human GH gene linked to either the mouse metallothionein or the chicken β -actin promoter (Lu *et al.*, 1992). Gross *et al.* (1992) reported some increase in the growth rate of northern pike following the transfer of the bovine GH gene linked to the RSV promoter. In a study by

Inoue *et al.* (1993) rainbow trout developing from eggs microinjected with an avian chimeric promoter and rainbow trout GH cDNA construct were 10 to 20% heavier than controls. A 70% increase in the growth of zebrafish was obtained by the transfer of a gene construct containing the RSV promoter and the coho salmon GH cDNA clone (Zhao *et al.*, 1993). The transfer of a human cytomegalovirus enhancer-promoter and the tilapia GH cDNA construct into tilapia, resulted in transgenic F₁ progeny 82% heavier than controls at 9 months old (Martinez *et al.*, 1996).

In contrast to the above studies, significant growth enhancement of salmonids has been achieved by the transfer of GH gene constructs where all the genetic elements are of piscine origin (Du *et al.*, 1992a; Devlin *et al.*, 1994, Devlin *et al.*, 1995). One such construct (opAFPGHc) consists of an ocean pout (*Macrozoarces americanus*) antifreeze protein gene (opAFP) promoter, a chinook salmon GH cDNA clone, and the opAFP polyadenylation and transcriptional termination signals (Du *et al.*, 1992a). When opAFPGHc DNA was transferred into Atlantic salmon, transgenic fish were generated with an average weight at one year 6 times greater than their sibling controls. Similar results were obtained with this construct in coho salmon, chinook salmon, rainbow trout and cutthroat trout (*Oncorhynchus clarki*) (Devlin *et al.*, 1995). The transgenic coho salmon showed an average increase of greater than 10-fold at 15 months old, and some individuals were 30 times heavier than controls. The use of electroporated sperm to produce growth enhanced transgenic loach by the transfer of opAFPGHc DNA has also been reported (Tsai *et al.*, 1995). After 161 days the group of loach resulting from electroporated sperm were 2.3 times heavier than the control group.

The gene construct used for this study, OnMTGH1, contains the sockeye salmon (*Oncorhynchus nerka*) growth hormone gene driven by the metallothionein promoter from the same species. When transferred into coho salmon by egg microinjection transgenics with significantly increased growth rates resulted (Devlin *et al.*, 1994). Most individuals found to contain the introduced GH gene exhibited the enhanced growth phenotype. At 12 months post fertilisation these fish had an average weight 11 times greater than normal fish, and the largest transgenic salmon was 37 times heavier than controls.

The transfer of genes into salmonids has been successfully accomplished by microinjecting eggs prior to first cleavage (Fletcher *et al.*, 1988; Devlin *et al.*, 1994) or at the one cell stage (Guyomard *et al.*, 1989; Rokkones *et al.*, 1989; Penman *et al.*, 1990). Microinjection of salmon eggs carried out by Dr. Robert Devlin and colleagues, at the Department of Fisheries and Oceans (DFO) Canada, West Vancouver Laboratory, have yielded gene transfer efficiencies of 4-6% in coho salmon (Devlin *et al.*, 1994; Devlin *et al.*, 1995) and approximately 1% in chinook salmon (Robert Devlin, personal communication). A parallel experiment was set up at the DFO laboratory to directly compare salmon sperm electroporation with the previously successful gene transfer method of egg microinjection. The OnMTGH1 construct was transferred into chinook and coho salmon, and the salmon were grown to 10 months old in the contained facilities available at the DFO. This allowed the screening of large numbers of individuals by monitoring their growth and gave further information on the persistence of DNA transferred by electroporated salmon sperm. This was important as only 12 week old fry had been analysed previously due to the lack of contained facilities available for long term culture.

Factors such as transfer efficiency, the number of growth enhanced fish produced, and the amount, fate and persistence of the introduced DNA were compared. Additionally, in order to determine the effects of electroporation on salmon sperm from different stocks and species, the viability of the shocked sperm was assessed.

5.2 Materials and Methods.

For the purpose of this study it was necessary to visit the West Vancouver laboratory during the 1994 salmon spawning season (October to November) and to return after 8 months in July 1995 to analyse the resulting groups of salmon. During the second visit (24 July to 18 August 1995) the fish were assessed for growth enhancement and fin samples taken for DNA analysis. DNA extraction and PCR analysis was carried out in Vancouver and some DNA samples were transported back to New Zealand for Southern analysis.

5.2.1 Gene construct.

The growth promoting gene construct used in this study, pOnMTGH (Figure 5.1), consists of the sockeye salmon metallothionein-B promoter fused to the full length type-1 sockeye salmon growth hormone gene (Devlin *et al.*, 1994).

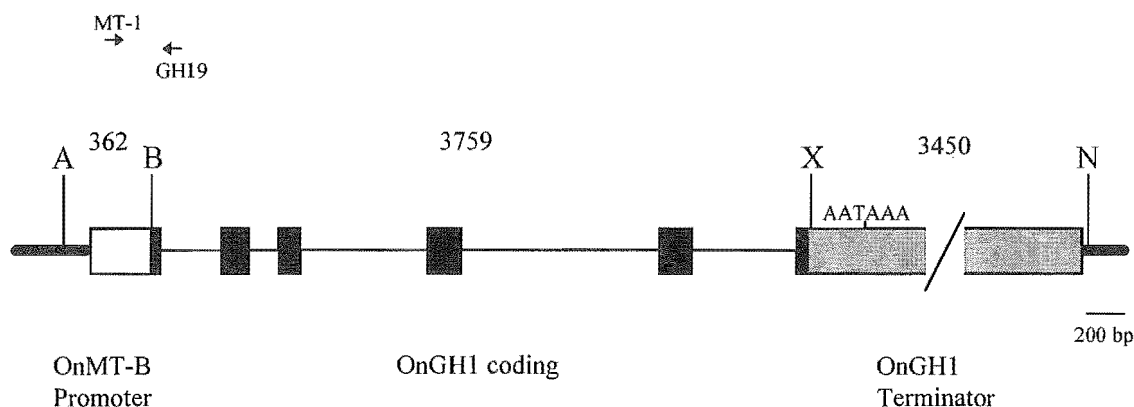


Figure 5.1. Schematic map of the OnMTGH1 gene construct. The OnMTGH1 construct (Devlin *et al.*, 1994) contains the sockeye salmon (*Oncorhynchus nerka*) metallothionein-B (OnMT-B) promoter (□, Chan and Devlin, 1994) and the full length type-1 sockeye salmon growth hormone gene (Devlin, 1993), including the six exons (■) and terminator sequence (■). The size of the different components is indicated in bp. The position of the binding sites of the oligonucleotides used to detect OnMTGH1 DNA (MT-1, and GH19) and the cutting sites for the restriction endonucleases *Apa*I (A), *Bam*HI (B), *Not*I (N), and *Xba*I (X) are indicated.

5.2.2 Isolation of plasmid DNA.

Plasmid DNA was isolated using an alkaline lysis protocol followed by caesium chloride gradient centrifugation. *Escherichia coli* cells transformed with pOnMTGH1 DNA were grown in 500 ml NZCYM media (NZ amine (20 g), NaCl (10 g), bacto-yeast extract (10 g), magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (4g), made up to 1 litre with water, pH 7) containing 50 $\mu\text{g/ml}$ ampicillin and incubated overnight at 37°C in a shaking incubator. The bacterial cells were then recovered by centrifugation at 5000 rpm (Sorvall, GSA rotor) for 10 minutes at 4°C. The cell pellet was resuspended in 10 ml of a cell lysis solution (5 mg/ml lysozyme, 50 mM glucose, 25 mM Tris-HCl, pH 8, 10 mM EDTA) and

incubated at room temperature for 5 minutes. 20 ml of alkaline lysis solution (40 ml of 1 M NaOH, 10.3 ml 20% SDS, 200 ml H₂O) was added and the suspension mixed until lysis was achieved. Following a 10 minute incubation on ice, 15 ml of ice-cold high salt solution (90 ml 5 M KOAc, 18 ml glacial acetic acid, 42 ml H₂O) was added to precipitate the bacterial chromosomal DNA. After incubation on ice for a further 10 minutes, the mixture was centrifuged at 15,000 rpm (Sorvall, SS34 rotor) for 30 minutes at 4°C and the supernatant filtered through a double layer of Kimwipes. The remaining nucleic acid was precipitated by the addition of 0.6 volumes of isopropanol. After centrifugation at 2,500 rpm (Beckman GS-6R, swinging bucket rotor) for 5 minutes, washing in cold 70% ethanol, and drying, the DNA was resuspended in TE8 (8 ml to every 500 ml of culture). Caesium chloride and ethidium bromide were added to give final concentrations of 1 g/ml and 1 mg/ml respectively. Following incubation in the dark for 15 minutes at room temperature and centrifugation at 3,700 rpm (Beckman GS-6R, swinging bucket rotor) for 10 minutes, the supernatant was transferred into ultracentrifuge tubes. Ultracentrifugation was carried out at 45,000 rpm (Beckman L8-M) for 48 hours at 25°C. The supercoiled plasmid DNA band was removed from the tube by side puncture with a needle and syringe. The ethidium bromide was removed by several extractions with isoamyl alcohol and the DNA precipitated by the addition of 2.5 volumes of 95% ethanol. After centrifugation at 3,700 rpm (Beckman GS-6R, swinging bucket rotor) for 30 minutes, washing in cold 70% ethanol, and drying, the DNA was resuspended in TE8.

5.2.3 Restriction enzyme digestion of plasmid DNA.

Digestion of plasmid DNA with restriction endonucleases (BRL) was carried out as outlined in section 2.2.7. Small scale double digests with *Apal* and *BamHI*, and *Apal* and *NotI* were carried out to confirm the identity of the plasmid (Figure 5.1). A large scale digest with *Apal* and *NotI* (0.5 Units of enzyme per mg of DNA) was employed to excise the insert from the pOnMTGH1 DNA (80 ng/ml).

5.2.4 Isolation of the OnMTGH1 insert DNA.

The OnMTGH1 insert DNA (7.57 kb) was separated from the vector DNA (2.98 kb) by electrophoresis and isolated by electroelution into dialysis tubing (Maniatis *et al.*, 1982). After the digested plasmid DNA fragments were separated by electrophoresis in a horizontal agarose gel (0.8% in $1 \times$ TBE), the region containing the band of interest was excised from the gel and sealed into a piece of dialysis tubing containing $1 \times$ TBE. Current was applied to run the DNA out of the agarose slice, and the buffer containing the DNA fragment removed from the tubing, which was further rinsed with TE8. The buffer containing the DNA was pooled and extracted with an equal volume of TE8 buffered phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 7,000 rpm (Sorvall, SS34 rotor) for 15 minutes. The aqueous phase was extracted with chloroform/isoamyl alcohol (24:1) and the DNA precipitated by the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and two volumes of ethanol. The DNA was recovered by centrifugation at 10,000 rpm (Sorvall, SS34 rotor) for 30 minutes, washed with ice cold 70% ethanol, dried and resuspended in TE8. The DNA concentration was determined spectrophotometrically as outlined in section 2.2.5. From an initial digestion of 4 mg of plasmid DNA a yield of 1.8 mg of insert DNA was obtained.

5.2.5 Electroporation of sperm.

The electroporation equipment was transported from New Zealand to Vancouver to allow the previously optimised parameters to be used to test the transfer of OnMTGH1 DNA. Sperm were electroporated using a method similar to that outlined in section 2.2.3. Half a ml of milt (approximately 2×10^{10} sperm) was diluted in a 1:1 ratio with $1 \times$ HBS containing pOnMTGH1 DNA. Electroporation was carried out with a Cell-Porator electroporation system (BRL) and the sperm incubated on ice for 10 minutes before being used to fertilise 1200 chinook or coho salmon eggs. The eggs and milt were collected from chinook salmon at the Big Qualicum hatchery on Vancouver Island, and from coho salmon at the Capilano hatchery, North Vancouver. The milt from one male chinook and coho salmon was used for the fertilisations of all eggs in each species. The eggs from 5

chinook, and 8 coho female salmon were pooled separately and mixed prior to the 1200 being set aside for each individual group. The percentage of motile sperm after incubation on ice was estimated upon activation with ovarian fluid.

Table 5.1 summarises the treatments and conditions applied to chinook salmon sperm prior to fertilisation. Most treatments were set up with the OnMTGH1 insert. However, to determine if the presence of the vector sequence affected expression of the growth hormone gene, pOnMTGH1 linearised with *NotI* was also used (treatment 12). Expression from the more easily obtained linearised plasmid DNA had not been previously tested. DNA concentrations of 20 or 200 µg/ml were used and the sperm were exposed to 1000 V/cm shocks of a single pulse of 18.6 ms or two pulses of 27.4 ms. Replicate treatments were set up to test the consistency of the results.

To evaluate the reproducibility of the results in another species, ten treatments were set up using coho salmon sperm (Table 5.2). Due to a limit on the amount of OnMTGH1 insert DNA that could be isolated, linearised plasmid DNA was used in six treatments. The plasmid was linearised with *ApaI* or *NotI*, both of which have cutting sites at one end of the OnMTGH1 insert (Figure 5.1). Both types of linearised plasmid DNA were used in case the presence of vector DNA adjacent to either end of the insert affected expression. In order to determine if gene transfer using electroporated sperm could be repeated using an alternative electroporation apparatus one sperm sample was shocked using a BioRad Gene Pulser. Like the Cell-Porator this machine generates pulses with exponential decay and, as with the other treatments, electroporation cells with a distance of 4 mm between the electrodes were used. The highest settings on the Gene Pulser of voltage (450) and capacitance 960 (µF) were used giving a pulse of 1125 V/cm × 15 ms. This was as similar to the pulses obtained with the Cell-Porator that could be obtained with this equipment. Double pulses with the Gene Pulser caused arcing between the electrodes and thus could not be used.

Table 5.1. Conditions used for the electroporation of chinook salmon sperm. OnMTGH1 insert DNA was used for all treatments except number 12, in which *NotI* linearised pOnMTGH1 DNA was electroporated with the sperm. NS = not screened.

Treatment number	Electroporation conditions: V/cm × ms	Number of pulses	DNA concentration (mg/ml)	Estimated sperm motility after electroporation (%)	Number of embryos surviving to the eyed stage from 1200 eggs	Proportion of positives by PCR screening of 11 day old embryos
C	Control	-	-	100	1061	0/9
1	No shock	-	200	100	1074	0/12
2	No shock	-	20	100	1008	0/15
3	1000 × 18.6	1	200	90	830	4/13
4	1000 × 18.6	1	200	95	1139	1/9
5	1000 × 18.6	1	20	95	1080	3/10
6	1000 × 18.6	1	20	95	973	0/6
7	1000 × 27.4	2	200	15	289	5/5
8	1000 × 27.4	2	200	10	102	NS
9	1000 × 27.4	2	20	15	361	4/5
10	1000 × 27.4	2	20	15	315	NS
11	1000 × 27.4	2	200	10	339	NS
12	1000 × 27.4	2	200	10	228	NS

Table 5.2. Conditions used for the electroporation of coho salmon sperm. The Cell Porator electroporation apparatus was used for all treatments except for number 16, in which the sperm were electroporated with the BioRad Gene Pulser (BGP). Sperm were electroporated with OnMTGH1 insert DNA (Insert), or with pOnMTGH1 plasmid DNA linearised with either *NotI* (*NotI*) or *ApaI* (*ApaI*). NS = not screened.

Treatment number	Electroporation conditions: V/cm × ms	Number of pulses	DNA type	DNA concentration (µg/ml)	Estimated sperm motility after electroporation (%)	Proportion of positives by PCR screening of 11 day old embryos
C2 (13)	Control 2	-	-	-	100	0/5
14	No shock	-	Insert	200	100	3/7
15	1000 × 27.4	2	Insert	200	15	3/5
16 (BGP)	1125 × 15	1	Insert	200	75	6/8
17	No shock	-	<i>ApaI</i>	200	100	NS
18	1000 × 27.4	2	<i>ApaI</i>	200	20	7/7
19	1000 × 27.4	2	<i>ApaI</i>	200	20	NS
20	No shock	-	<i>NotI</i>	200	100	NS
21	1000 × 27.4	2	<i>NotI</i>	200	15	7/7
22	1000 × 27.4	2	<i>NotI</i>	200	10	NS

5.2.6 Microinjection.

In order to compare salmon sperm electroporation with the previously successful technique of egg microinjection, OnMTGH1 DNA was injected, after fertilisation, into 290 chinook salmon eggs and 139 coho salmon eggs. The procedure was carried out by Timothy Yesaki. Eggs were fertilised in ice cold Ginsburg's saline (110 mM NaCl, 3.4 mM KCl, 2.4 mM NaHCO₃, 2.7 mM CaCl₂; Ginsburg, 1963). Under these conditions egg activation does not occur and the chorion remains soft enough to be penetrated with a microinjection needle. 1-2 nl of OnMTGH1 insert DNA (100 ng/ml in 1mM Tris, 0.1 mM EDTA 0.5% phenol red, pH 7.2) were injected through the chorion and vitelline membranes into the germinal disc region using a 5-10 µm needle with a nitrogen gas driven injection system (Eppendorf, model 5242). After injection the eggs were transferred to well water to initiate development. The chinook and coho salmon resulting from the microinjected eggs were reared until hatching, but only the coho salmon were analysed at the 10 month old stage.

5.2.7 Salmon rearing.

Salmon embryos were maintained in Heath trays supplied with 12 ± 1 °C well water (coho 9 ± 1 °C). The fry were transferred at the swim up stage (3 months post fertilisation) into 200 litre circular tanks supplied with 8.5 ± 1.5 °C well water, where they were grown to ten months old. The rearing of the salmon and the transfer of groups was carried out by the staff of the DFO West Vancouver molecular genetics laboratory.

5.2.8 Isolation of DNA from embryos.

Genomic DNA was extracted for PCR analysis from embryos using a salting out procedure. The embryos were dissected into ice cold Ginsburg's saline and the chorion removed. To remove the yolk the embryos were washed twice in ice cold Ginsburg's saline. If individual embryos were too small to manipulate, this washing was accomplished by spinning at 3,400 rpm (microfuge) for 5 minutes at 4°C, and then removing the supernatant. Each embryo was transferred into 50 µl of proteinase K

digestion buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS) containing 200 µg/ml proteinase K. Digestion was carried out at 50°C for at least 1 hour with intermittent mixing. To precipitate the protein, 17 µl of a saturated NaCl solution was added and the protein and debris were removed by centrifugation at 12,000 rpm (microfuge) for 15 minutes. To assist the precipitation of the embryonic DNA, 1 µg of salmon genomic DNA was added to the supernatant. The DNA was then precipitated by the addition of two volumes of ethanol, followed by incubation at room temperature for 16 hours. The DNA was recovered by centrifugation at 12,000 rpm (microfuge) for 30 minutes, washed with ice cold 70% ethanol, dried and dissolved in 25 µl of TE8. A small number of samples were subsequently purified for Southern analysis. The volume of these samples was increased to 500 µl with TE8, then extracted with organic solvents, precipitated and dissolved in TE8 as described in section 3.2.5.

High quality genomic DNA was extracted from some embryos exclusively for Southern analysis. After dissection and washing in Ginsburg's saline these were digested at 50°C in 500 µl of proteinase K digestion buffer containing 200 µg/ml proteinase K. To assist the precipitation of the embryonic DNA, 1 µg of salmon genomic DNA was added. The samples were extracted with organic solvents, precipitated and dissolved in TE8 as described in section 3.2.5.

5.2.9 Preparation of crude DNA from salmon.

The salmon groups were reared up to 10 months old. At this point all the fish were weighed and a tissue sample (adipose fin) was taken from a sample of the fish to be screened by PCR. These fish were anaesthetised in 100 mg/l tricane methane sulfonate, injected with a Passive Integrated Transponder tag and their adipose fin removed. A small piece of fin tissue was digested in 200 µl of K buffer (20 mM Tris, pH 8.3, 2.5 mM MgCl₂, 50 mM KCl, 0.5% Tween-20), containing 200 µg/ml proteinase K, at 56°C for 1 hour. The preparation was incubated at 95°C for 10 minutes, vortexed and centrifuged at 12,000 rpm (microfuge) for 2 minutes. Two µl of the supernatant was used for PCR

OnMTGH1 DNA), extraction control tubes were included to ensure any contamination with OnMTGH1 DNA was detected. These consisted of the same components as the other tubes but no fin tissue was added.

5.2.10 PCR screening.

PCR was used to amplify a 400 bp fragment of the OnMTGH1 construct using the two oligonucleotide primers MT-1 and GH19 (Figure 5.1) (see Appendix 1 for primer sequences and PCR conditions). The amplification was carried out in a 50 µl volume containing 2 µl of template DNA solution, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.001% gelatin, 0.2 mM each of the deoxyribonucleoside triphosphates (dNTPs); dTTP, dATP, dCTP and dGTP, one unit of *Taq* polymerase (BRL) and 50 pmol each of two primers. Thermal cycling was carried out with a Perkin Elmer Thermal Cycler 480 as outlined in section 2.2.7. The products were electrophoresed in 1% agarose gels in 1 × TBE for 1 hour at 120 V (100 mA) using a horizontal gel apparatus. The DNA was stained in 5 µg/ml ethidium bromide and viewed under ultra-violet light (254 nm).

As a control, the 311 bp MCH fragment was amplified from at least 10% of samples from each series of extractions, as outlined in section 2.2.7. The MCH fragment was successfully amplified from all of the crude fin extractions tested (Figure 5.2), indicating that amplifiable DNA was present in these samples.

5.2.11 Southern hybridisation analysis.

The procedures for electrophoresis of DNA in preparation for Southern blotting, probe preparation and hybridisation, were the same as outlined in sections 3.2.8 and 3.2.9. The sockeye salmon GH gene fragment excised from pOnMTGH1 with *Bam*H I and *Not*I (Figure 5.1) was used as the hybridisation probe. This DNA fragment was isolated and purified using agarose electrophoresis and the Prep-A-Gene kit as outlined in section 3.2.10.

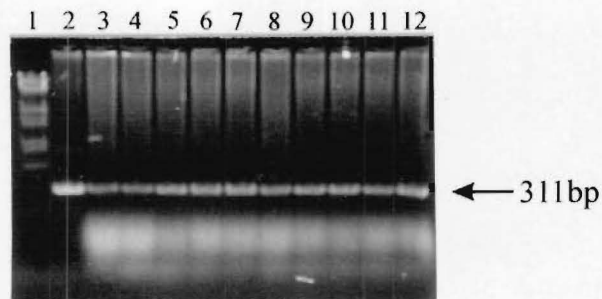


Figure 5.2. Example of the melanin concentrating hormone gene PCR products amplified from embryo DNA samples. Lane 1: lambda DNA digested with *EcoR* I and *Hind* III (Fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125) ; 2: positive control, 311 bp MCH product amplified from salmon genomic DNA(0.2 μ g); 3 to 12: DNA extracted from salmon embryos used as the template.

5.3 Results.

5.3.1 Salmon rearing.

Due to silting of the water supply to the Heath trays in the hatchery, high mortalities of salmon occurred just prior to hatching. This resulted in the loss of several groups and high mortalities in other groups. The groups and number of fish surviving to 10 months old are indicated in Table 5.3. Due to the effect of environmental conditions on the number of fish surviving to ten months post fertilisation it is not possible to compare the survival of the groups or relate this to the treatment of the sperm. Just prior to weighing the fish, a large proportion of the fish in treatment 21/22 died due to a water flow problem. Up to this point the number of fish in this group was similar to that of treatment 18/19. The microinjected coho group was not affected by the silting in the hatchery and the survival at 10 months old was 82% (114/139).

5.3.2 Sperm viability.

To determine the effect of electroporation on the fertilising capabilities of electroporated sperm, sperm motility was estimated and survivorship of the chinook salmon groups to the eyed stage was assessed. Electroporation was found to decrease the motility of chinook salmon sperm (Table 5.1) and the extent of the reduction was dependant on the electroporation conditions. When one pulse of $1000 \text{ V/cm} \times 18.6 \text{ ms}$ was applied, the chinook salmon sperm motility was reduced to approximately 90 to 95%, compared to 100% when no electric pulse was applied. Two pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$ resulted in a motility of approximately 10 to 15%. The motility of coho salmon sperm was also reduced to a similar level (10 to 20%) when electroporated with two pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$ (Table 5.2).

The number of chinook salmon surviving from 1200 eggs to the eyed stage (Table 5.1) was also affected by the electroporation conditions (Figure 5.3). When one $1000 \text{ V/cm} \times 18.6 \text{ ms}$ pulse was used, the number surviving decreased slightly. When two pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$ were used, 272 eyed eggs developed on average, compared to an average of 1048 obtained when non-electroporated sperm were used. The number of chinook salmon surviving to 10 months old was affected by the unfortunate loss after hatching and therefore cannot be compared with the electroporation conditions.

Table 5.3. Analysis of 10 month old chinook and coho salmon. In all treatments sperm were electroporated with 2 pulses of 1000 V/cm \times 27.4 ms using the Cell Porator, except for treatment number 16 where one pulse of 1125 V/cm \times 15 ms was applied by the Gene Pulser. The number of individuals weighing greater than two standard deviations (2SD) above the mean is indicated for each group. The number of salmon screened by PCR for the presence of OnMTGH1 DNA in fin and blood samples is given for each treatment. NA = not applicable.

Treatment number	Species	DNA type	DNA concentration (μ g/ml)	Number of fish weighed	Number weighing 2SD above the mean (%)	Number of fish screened by PCR	
						Fin	Blood
8	chinook	Insert	200	73	2 (2.7)	73	0
9	chinook	Insert	20	272	5 (1.8)	12	0
10	chinook	Insert	20	16	0 (0)	7	0
11	chinook	Insert	200	122	2 (1.6)	122	4
12	chinook	<i>NotI</i>	200	119	5 (4.2)	14	0
15	coho	Insert	200	353	12 (3.4)	22	2
16	coho	Insert	200	880	29 (3.3)	19	0
18/19	coho	<i>Apal</i>	200	1204	29 (2.4)	19	0
21/22	coho	<i>NotI</i>	200	261	4 (1.5)	207	6
Microinjection	coho	Insert	NA	114	21 (18.4)	0	114

The number of viable eyed coho salmon embryos was not assessed. However, when the groups of 10 month old fish were weighed, and despite rearing problems, up to 1200 fish were found to have survived from the 2400 eggs fertilised with sperm electroporated with two pulses of 1000 V/cm \times 27.4 ms (Table 5.3).

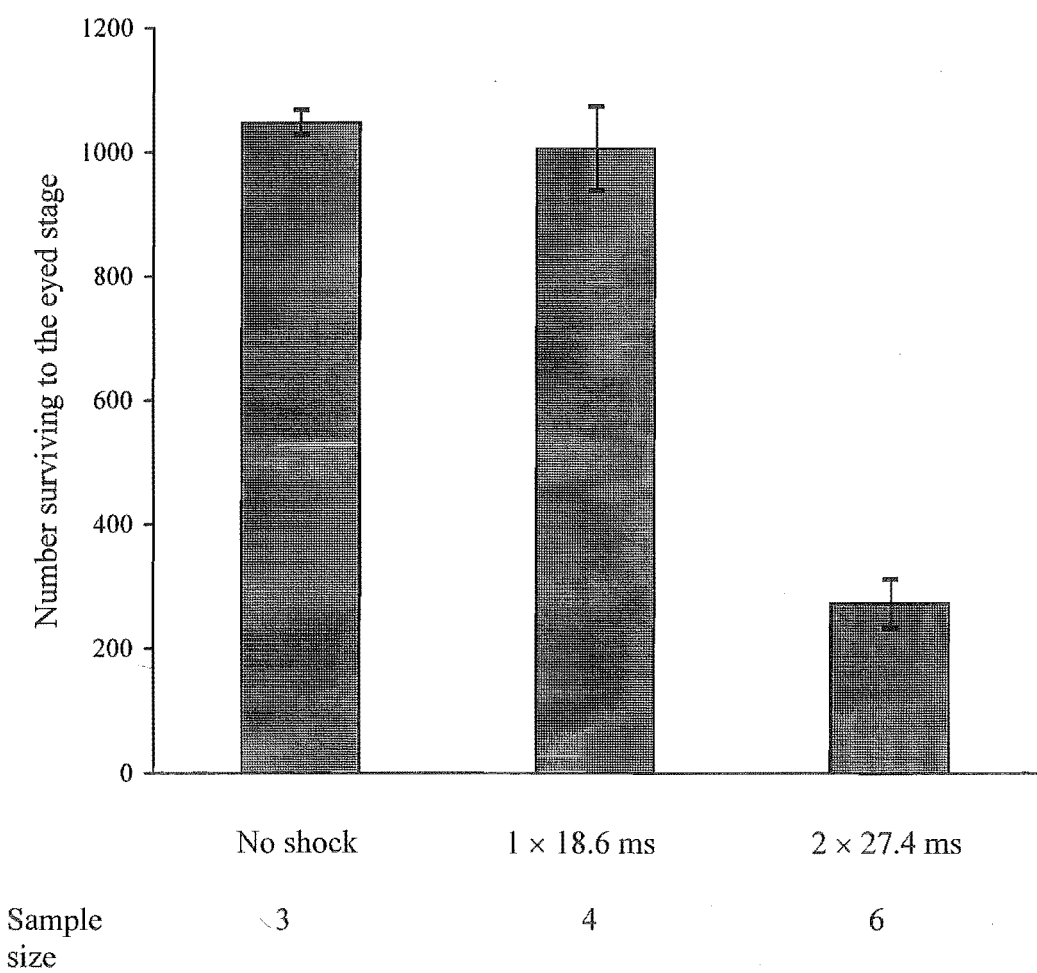


Figure 5.3. The effect of the sperm electroporation conditions on the survival of chinook salmon to the eyed stage. The average number of chinook salmon surviving to the eyed stage resulting from 1200 eggs fertilised with unshocked sperm (No shock), one 1000 V/cm \times 18.6ms pulse (1 \times 18.6 ms), or two 1000 V/cm \times 27.4ms pulses (2 \times 27.4 ms) is presented. The number of individual groups analysed is indicated (sample size).

5.3.3 Detection of introduced OnMTGH1 DNA in embryos.

5.3.3.1 PCR screening.

In order to make an early assessment of the experiment, preliminary screening was carried out on embryonic fish. Using the salting out method, DNA was extracted from 11 day old (stage 18, eyes just pigmented) embryos and screened for the presence of OnMTGH1 DNA by PCR. Figure 5.4 shows an example of the results generated by this screening.

The proportion of chinook salmon embryos found to contain the transferred DNA is shown in Table 5.1. No OnMTGH1 DNA was detected in embryo DNA samples when non-electroporated chinook salmon sperm were used. When sperm were exposed to two pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$, PCR product was obtained in 5 out of 5 samples when $200 \mu\text{g/ml}$ of DNA were mixed with the sperm, and in 4 out of 5 embryo DNA samples when the DNA concentration was $20 \mu\text{g/ml}$ (Table 5.1 and Figure 5.4b). OnMTGH1 DNA was also detected when one shock of $1000 \text{ V/cm} \times 18.6 \text{ ms}$ was used, but the proportion of positive embryos detected was lower than in the two pulse treatments (Table 5.1). There was no clear relationship between the DNA concentration and the proportion of positive embryos in the one pulse treatments. DNA samples from embryos resulting from chinook salmon eggs microinjected with OnMTGH1 DNA were also screened by PCR. Transferred DNA was detected in 4 out of the 8 11 day old fish analysed (Figure 5.4).

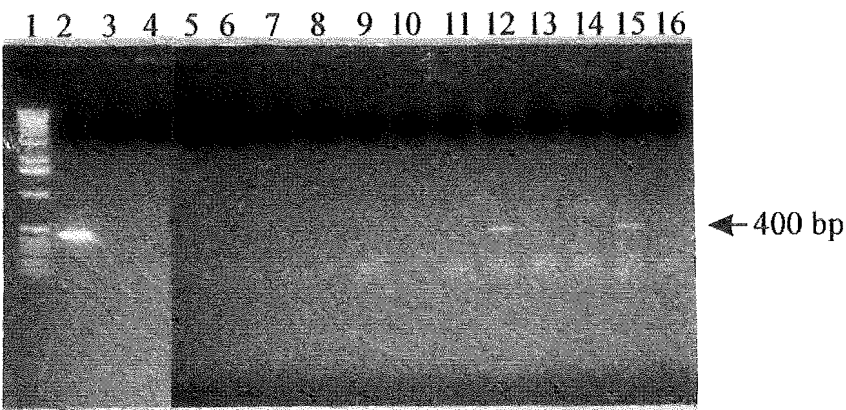
The number of coho salmon embryos found to contain the transferred DNA is shown in Table 5.2. In this species, even when non-electroporated sperm were used, OnMTGH1 DNA was detected in 3 out of the 7 samples screened. When sperm were electroporated with pOnMTGH1 DNA ($200 \mu\text{g/ml}$) linearised with either *ApaI* or *NotI*, transferred DNA was detected in all 7 embryos tested. Therefore successful gene transfer into coho salmon embryos was achieved with both types of DNA (insert only and linearised plasmid) and did not appear to be inhibited by the inclusion of the vector DNA. Gene transfer into the embryos was also observed when the sperm were electroporated with the Gene Pulser apparatus.

Figure 5.4. Example results of screening for the presence of OnMTGH1 DNA in embryo DNA by PCR. The DNA analysed was extracted from 11 day old chinook salmon embryos.

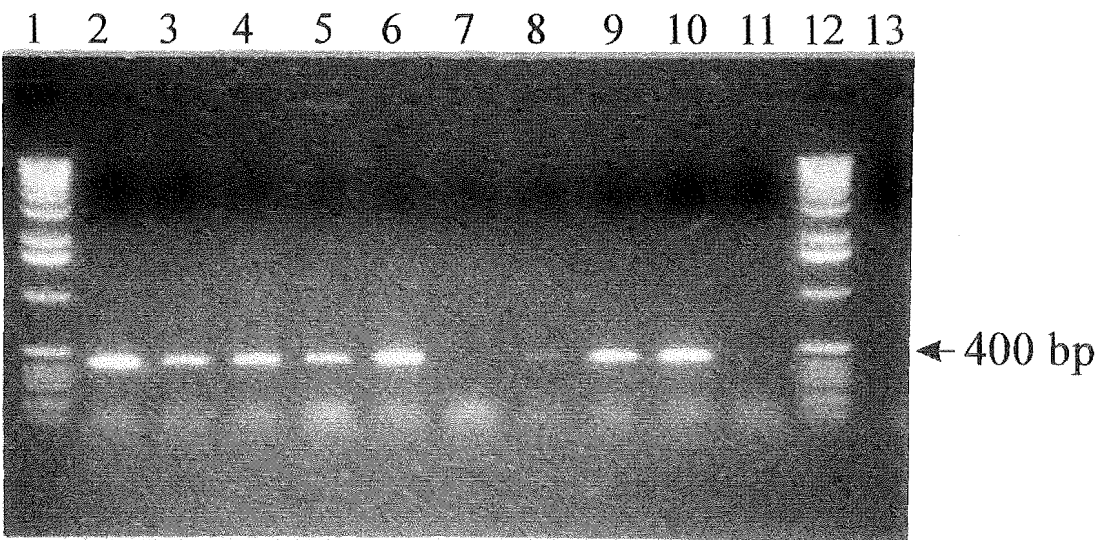
(a) Lane 1: 1 kb ladder (Fragment sizes (bp): 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 506, 517, 396, 344, 293, 220, 201, 154, 134, 75) ; 2: positive control, 400 bp product amplified from OnMTGH1 DNA (1 pg); 3 and 4: negative controls, extraction blanks; 5 to 8: control fish; 9 to 16: embryos resulting from eggs microinjected with OnMTGH1 DNA.

(b) Lanes 1 and 12: 1 kb ladder (Fragment sizes (bp): 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 506, 517, 396, 344, 293, 220, 201, 154, 134, 75); 2 to 6: embryos resulting from sperm electroporated with two 1000 V/cm \times 27.4 ms pulses in the presence of 200 μ g/ml of OnMTGH1 DNA; 7 to 11: embryos resulting from sperm electroporated with two 1000 V/cm \times 27.4 ms pulses in the presence of 20 μ g/ml of OnMTGH1 DNA; 13: water control reaction.

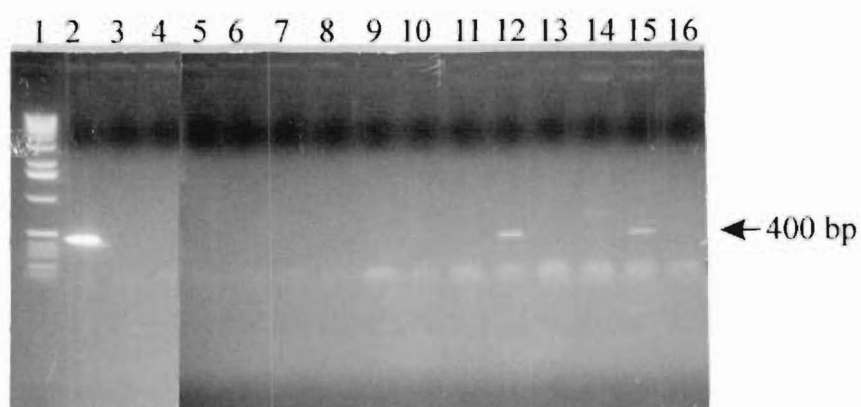
(a)



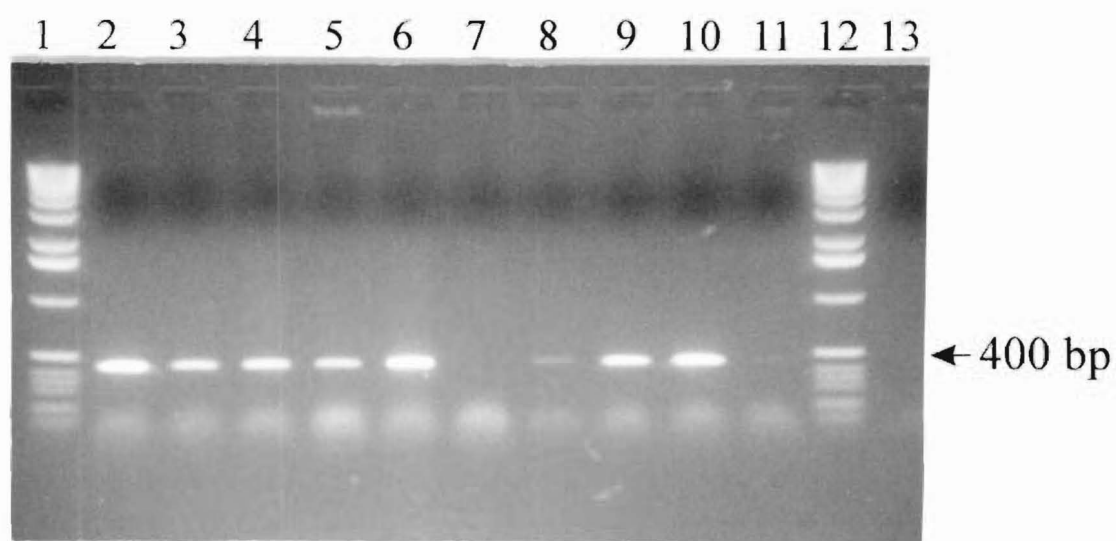
(b)



(a)



(b)



No OnMTGH1 was detected in: any of the extraction blanks, the control fish analysed, or the PCR water control tubes (see Figure 5.4), indicating that there was no contamination with OnMTGH1 DNA during the extractions or setting up of the PCR reactions.

5.3.3.2 Southern analysis of embryos.

Southern analysis was used in an attempt to determine the amount and fate of DNA transferred into the embryos by electroporated sperm and to compare this with DNA introduced by microinjection. DNA was extracted from 0.88, 1.8, 8 and 11 day old chinook salmon embryos (of embryonic stages: 16 to 32 cell; 7, late morula; 13, late gastrula; 18, eyes just pigmented) using the salting out method followed by organic extractions. All the resulting DNA samples were analysed by gel electrophoresis and Southern hybridisation using the salmon GH gene probe (Figures 5.5a and 5.5b). The treatments, number of embryos analysed, and the proportion found to contain introduced OnMTGH1 DNA are presented in Table 5.4.

In positive samples resulting from electroporated sperm (for example, lanes 13 to 17, Figure 5.5a) transferred DNA was detected as a single band, with a mobility identical to the 7.57 kb OnMTGH1 construct (lanes 1 and 2, Figure 5.5a). In contrast to the sperm transferred DNA, OnMTGH1 detected in the DNA from 2 of the 6 microinjected samples (lanes 7 and 11, Figure 5.5a), appeared as two bands with mobilities corresponding to 62.2 and 15.4 kb. Binding of the GH probe was also detected in the area of the gel corresponding to the undigested genomic DNA. This was most pronounced in Figure 5.5b and was detected in negative control fish (lanes 11 and 16, Figure 5.5b), as well in the experimental embryo DNA samples (lanes 4, 5, 7 to 10, 12 to 15, 17 and 18, Figure 5.5b). This represents hybridisation of the GH probe to the endogenous GH gene. The size range of salmon genomic DNA electrophoresed on these gels was approximately 19.4 to 31 kb.

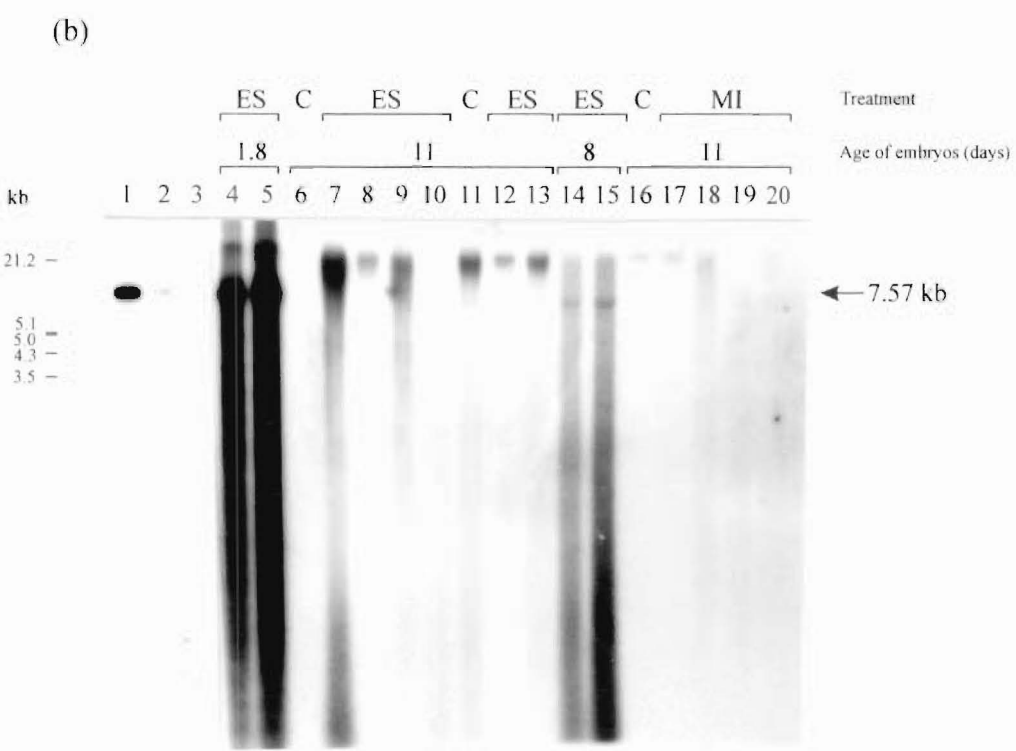
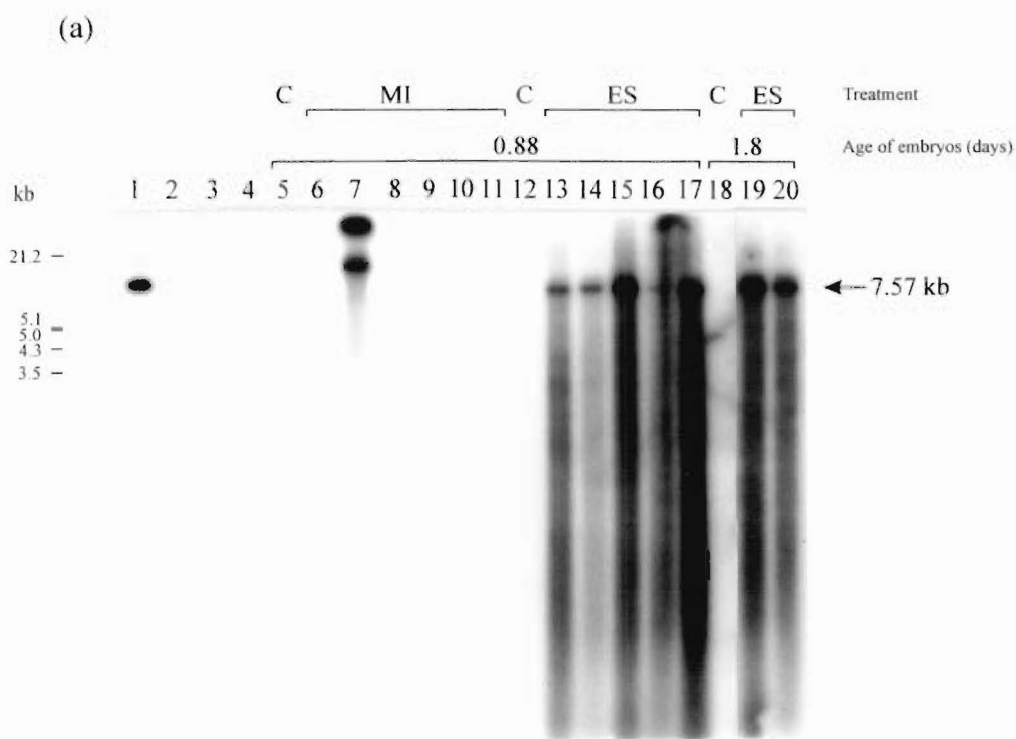
Table 5.4. Number and age of chinook salmon embryos analysed by Southern analysis. The proportion of individuals found to contain introduced OnMTGH1 DNA is indicated (see also Figure 5.5). NS = not screened.

Treatment	Electroporation conditions: V/cm × ms	Number of pulses	DNA concentration (µg/ml)	Age of embryos (days)			
				0.88	1.8	8	11
				Proportion of embryos in which OnMTGH1 DNA was detected			
Microinjection	-	-	-	2/6	NS	NS	0/4
3	1000 × 18.6	1	200	NS	NS	NS	1/1
7	1000 × 27.4	2	200	5/5	4/4	2/2	1/4
9	1000 × 27.4	2	20	NS	NS	NS	0/2

Figure 5.5 Detection of OnMTGH1 DNA in undigested chinook salmon embryo DNA by Southern analysis.

(a) Lane 1: OnMTGH1 insert DNA (20 pg); 2: OnMTGH1 insert DNA (2 pg); 3: OnMTGH1 insert DNA (0.2 pg); 4: 1 μ g of salmon genomic DNA (the same DNA solution added to the embryo DNA samples to assist in efficient precipitation during the extraction procedure); 5 to 20: DNA extracted from control embryos (C), embryos resulting from microinjected eggs (MI) or electroporated sperm (ES). In all the sperm electroporation samples analysed the condition used was two 1000 V/cm \times 27.4 ms pulses. The DNA concentration used in the sperm electroporation samples was 200 μ g/ml. The ages of the embryos analysed are indicated for each lane.

(b) Lane 1: OnMTGH1 insert DNA (20 pg); 2: OnMTGH1 insert DNA (2 pg); 3: OnMTGH1 insert DNA (0.2 pg); 4 to 20: DNA extracted from control embryos (C), embryos resulting from microinjected eggs (MI) or electroporated sperm (ES). In all the sperm electroporation samples analysed the condition used was two 1000 V/cm \times 27.4 ms pulses, except for the sample in lane 7, where one 1000 V/cm \times 18.6 ms pulse was used. The DNA concentration used in the sperm electroporation samples was 200 μ g/ml, except for those in lanes 12 and 13 where 20 μ g/ml of OnMTGH1 DNA was used. The ages of the embryos analysed are indicated for each lane.



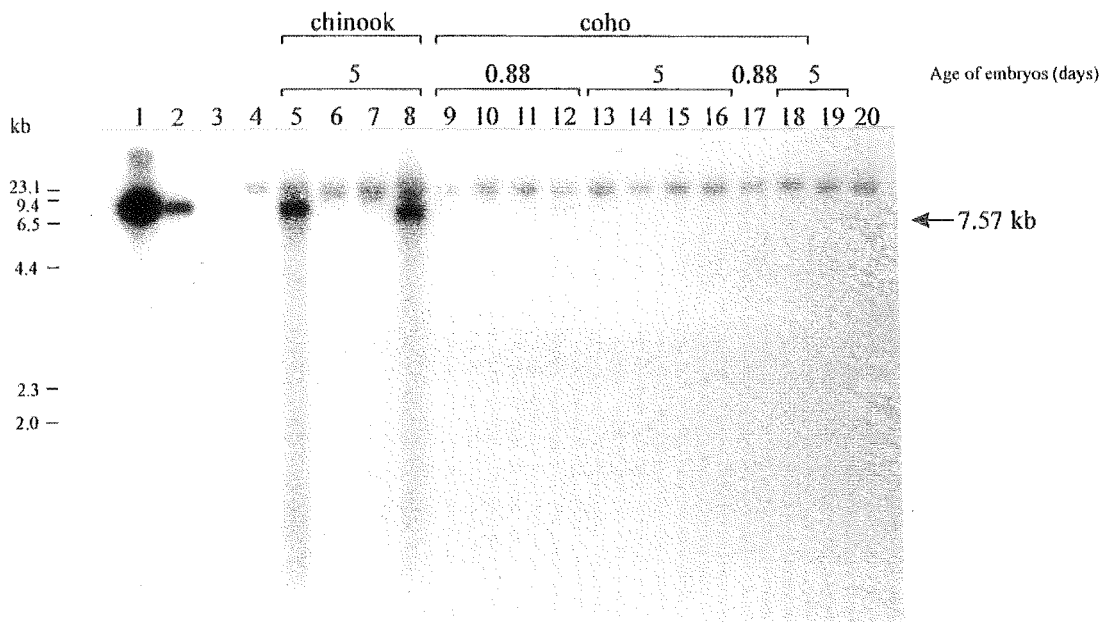
Due to the inconsistency of the salting out procedure the yield from the DNA extractions was variable. Therefore, conclusions could not be made about the amount of introduced DNA present in the embryos (all the DNA resulting from each embryo extraction was loaded in each lane). However, it is clear by comparison to control DNA lanes that at least 20 pg (approximately 2.4×10^6 copies) of OnMTGH1 is present in the 16 to 32 cell embryos resulting from both microinjection and sperm electroporation. No OnMTGH1 DNA was detected in DNA extracted from control fish (lanes 6 and 12, Figure 5.5a).

To determine the amount of DNA transferred into embryos by electroporated sperm more accurately, further DNA extraction and Southern analysis was carried out. Unfortunately no additional samples of microinjected embryos were available for analysis at this point. To ensure higher quality DNA and consistent yields, tissue digestion and organic extraction was carried out in a larger volume than for the salting out method. The previous results indicated that the sperm transferred DNA existed in the embryo in an unmodified linear form. However, the possibility that it may be existing as a multimer, with an identical mobility to linear DNA, could not be excluded. For this reason the samples were split into two equal portions. One half was digested with the restriction enzyme *XbaI* before being electrophoresed on replicate agarose gels and subjected to Southern analysis. Figure 5.6a and 5.6b shows the results of this analysis carried out on 0.88 day old coho salmon embryos (16 to 32 cell stage) and on five day old embryos (stage 9, beginning of gastrulation) of chinook and coho salmon. All the samples analysed resulted from sperm electroporated with 2 pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$ with $200 \mu\text{g/ml}$ of OnMTGH1 DNA. Introduced DNA was detected in 2 of the 4 chinook salmon embryos tested (lanes 5 and 8, Figure 5.6a and 5.6b). The amount of introduced DNA observed was equivalent to approximately 40 pg (approximately 4.8×10^6 copies) per embryo. The transferred DNA was detected as a single band with the same mobility as the 7.57 kb OnMTGH1 construct when undigested embryo genomic DNA was examined (Figure 5.6a). Introduced DNA was only detected faintly in one of the 21 hour old coho salmon embryos (lane 12, Figure 5.6a). When the embryo DNA was digested with *XbaI* the

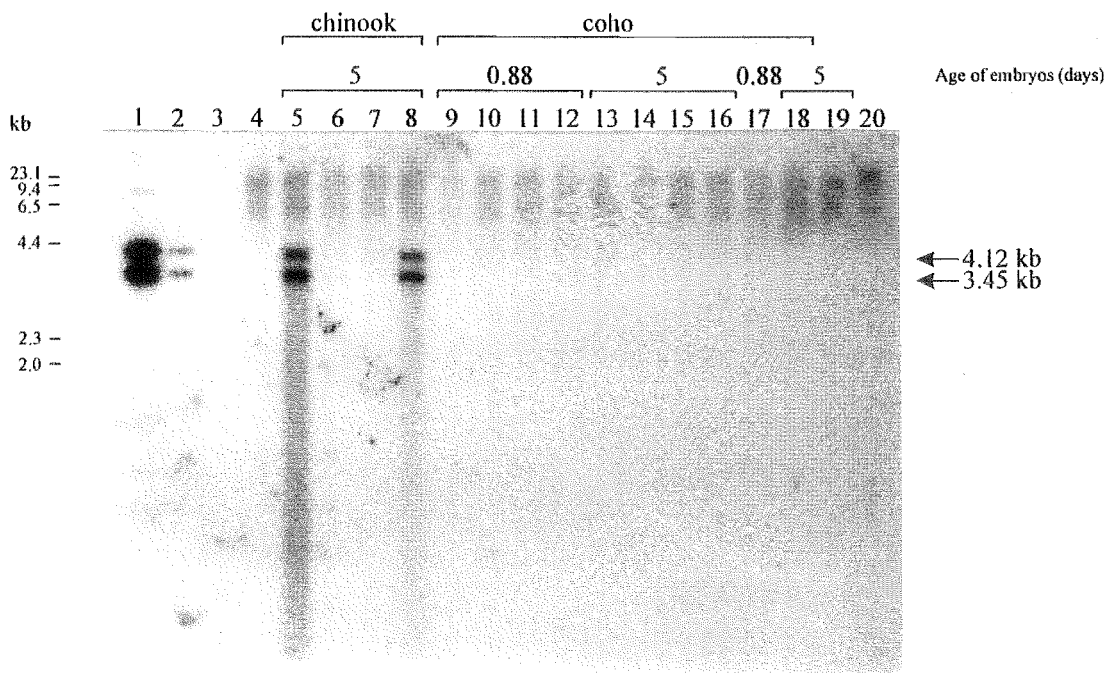
Figure 5.6 Detection of OnMTGH1 DNA in undigested and restriction endonuclease digested embryo DNA by Southern analysis. Identical samples were loaded on two separate agarose gels, except that those in (a) were undigested, and those in (b) were digested with *Xba*I.

Lane 1: OnMTGH1 insert DNA (20 pg); 2: OnMTGH1 insert DNA (2 pg); 3: OnMTGH1 insert DNA (0.2 pg); 4: 1 µg of salmon genomic DNA (the same DNA solution added to the embryo DNA samples to assist in efficient precipitation during the extraction procedure); 5 to 16: DNA samples from embryos resulting from sperm incubated with 200 µg/ml of OnMTGH1 DNA and exposed to two electric pulses of 1000 V/cm × 27.4 ms; 17 to 19: control embryo samples; 20: extraction blank. The age and species is indicated for each sample (lane 20: chinook).

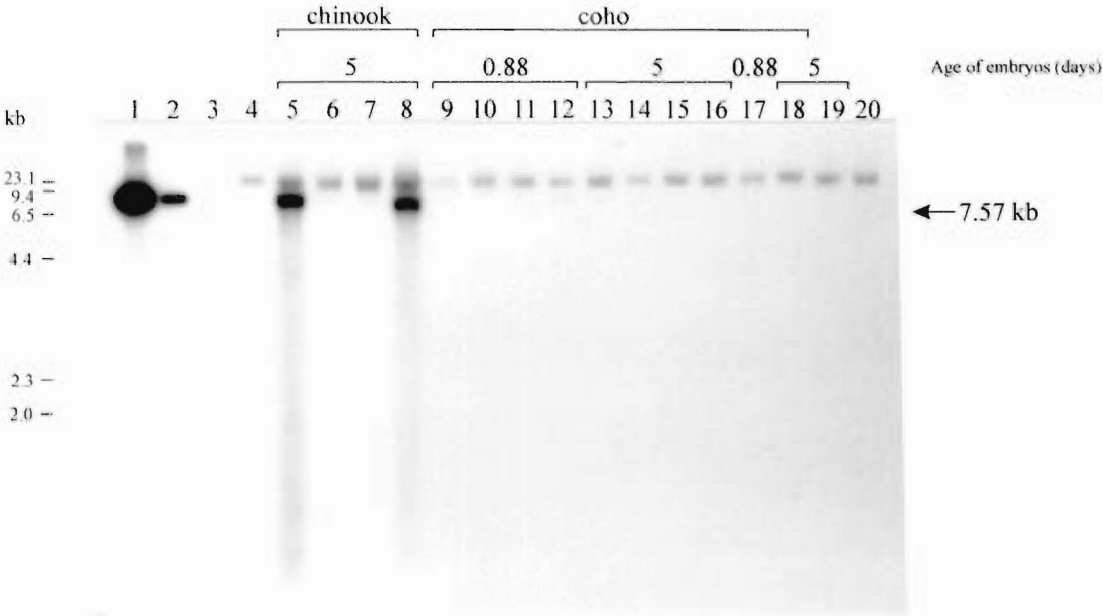
(a)



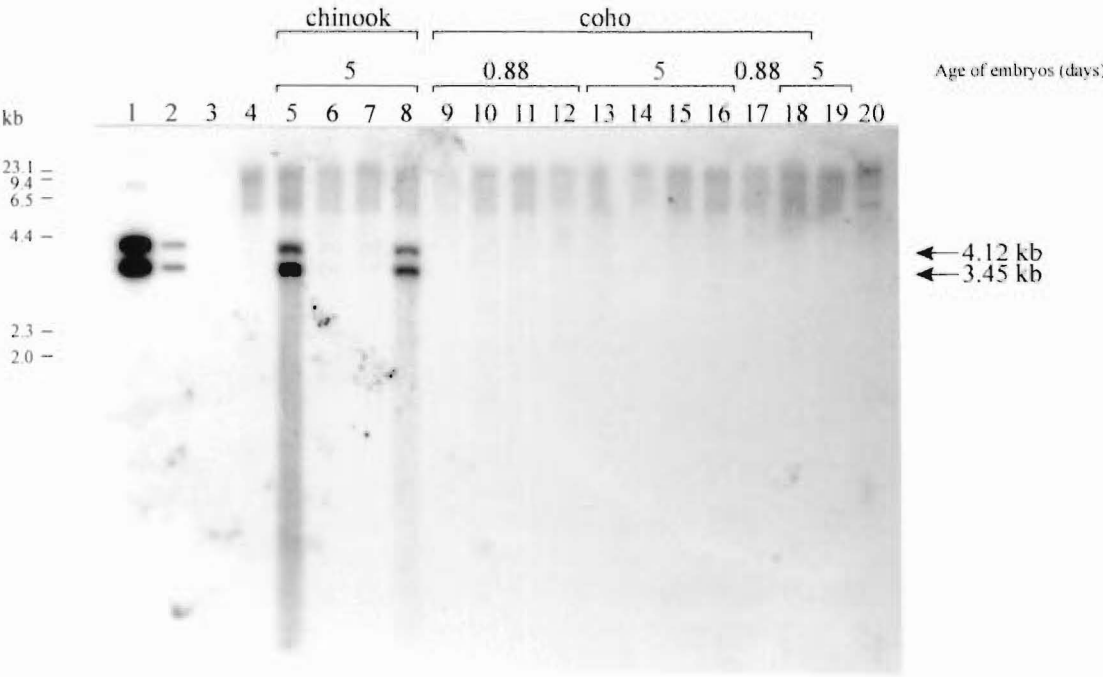
(b)



(a)



(b)



banding pattern was also identical to similarly treated OnMTGH1 DNA, with two bands of 4.12 and 3.45 kb being obtained (Figure 5.6b). Binding to the endogenous GH gene was similarly detected in control DNA (lane 4, Figure 5.6a and b), in control embryos (lanes 17 to 19, Figure 5.6a and b), and in experimental embryo lanes (lanes 5 to 16, Figure 5.6a and b). No OnMTGH1 DNA was detected in DNA extracted from control fish or extraction blanks.

5.3.4 Detection of OnMTGH1 DNA in fish by phenotype and PCR.

The embryos were grown to hatch and transferred into circular tanks for rearing up to 10 months. This enabled a large number of individuals to be screened for the presence of the OnMTGH1 construct on the basis of growth rate. Due to water quality problems in the hatchery only some of the original groups of fish survived. Table 5.3 shows the treatments that were assessed for enhanced growth. Due to a lack of rearing tanks the replicate coho treatments, in which linearised plasmid DNA was used, were pooled together (treatment 18 was pooled with 19, and 20 with 21).

After 10 months, all the fish were weighed. Due to variable mortalities caused by environmental factors, the treatments were reared at different densities. Therefore the average weights of the treatments were quite different and comparisons between these can not be made. There were no obviously growth enhanced individuals in the sperm electroporation treatments, but some groups did contain heavier individuals outside the normal weight distribution. In four of the groups the proportion with weights greater than two standard deviations (SD) above the mean exceeded 2.5% (see Table 5.3). Additionally, in three treatments the proportion of individuals weighing greater than 3SD above the mean was greater than 0.15 %: 0.28% (1/353), 0.58% (7/1204) and 0.38% (1/261) in treatments 15, 18/19 and 21/22 respectively. However, none of the larger individuals were found to be positive by fin PCR (see below). The frequency distributions of the weights of these salmon are presented in Figure 5.7 (a to i, presented at the end of this chapter, pages 156 to 163).

Fin samples from some of the fish were screened for the presence of OnMTGH1 DNA by PCR. The number of individuals in each treatment screened using this method is given in Table 5.3. All the remaining fish from the chinook salmon groups arising from sperm electroporated with 200 µg/ml of insert DNA (treatments 8 and 11: 2 pulses of 1000 V/cm × 27.4 ms), and 207 coho salmon from treatment 21/22 (200 µg/ml of *NotI* linearised pOnMTGH1 DNA, 2 pulses of 1000 V/cm × 27.4 ms) were PCR screened. Additionally, the largest 7 to 22 fish of the other remaining treatments were also analysed. No OnMTGH1 DNA was detected by PCR in any of the 495 fish analysed in this way.

Blood samples were obtained from twelve individuals whose fin tissue had been screened for the presence of introduced DNA. PCR analysis was carried out on crude preparations of these blood samples. The treatments and number of salmon analysed in this way are included in Table 5.3, and their weights (at 10 months old) are indicated on the weight frequency distributions (Figures 5.7d (page 158), 5.7f (page 159) and 5.7i (page 162)). As in the fin analysis, no OnMTGH1 DNA was detected by PCR in the blood from these salmon.

In contrast to the sperm electroporation treatments, growth enhanced salmon were observed when egg microinjection was used to transfer OnMTGH1 DNA. All 114 10 month old coho salmon, originating from microinjected eggs, were weighed and examined by PCR for the presence of the introduced DNA. Due to difficulties extracting crude DNA from adipose fin tissue, a crude preparation of DNA for the PCR analysis was extracted from blood samples. Figure 5.7(j) (page 163) shows the weight distribution of these fish. Of the 114 fish analysed, 18 heavier individuals were obtained with weights greater than 3SD above the mean of the normal sized fish. In fact, the 15 largest fish (13.2% of the group) had weights over 10SD above the mean weight of the normal sized fish. The mean weight of the majority of the fish was 8.9 g, while the larger individuals had an average weight of 198.4 g, the heaviest being 415 g. Twelve of the 18 larger fish were found to contain introduced DNA by PCR analysis of blood samples, and two normal sized fish were also shown to contain OnMTGH1 DNA (see Figure 5.7j (page 163)).

5.4 Discussion.

In agreement with the previous experiments presented in Chapter 3, electroporation was found to decrease the motility of chinook and coho salmon sperm. However, even when 1200 eggs were fertilised with electroporated sperm with the most reduced motility (using two pulses of longer duration) an average of 270 (23%) viable eyed chinook salmon eggs were produced, and up to 1204 (50%) coho salmon survived to 10 months post fertilisation. This is lower than the 82% survival obtained in this study when coho salmon eggs were microinjected, and the 80 to 90% survival rates obtained in other salmon microinjection experiments (Fletcher *et al.*, 1988; Shears *et al.*, 1992; Devlin *et al.*, 1995). However, as more eggs can easily be treated when using sperm for gene transfer, such mortalities are acceptable.

Evidence that electroporated sperm can successfully transfer the growth hormone gene construct into embryos of chinook and coho salmon was obtained. Although sample sizes were low, the PCR screening of 11 day old embryos indicated that electroporated sperm were more efficient at introducing DNA into embryos than microinjection. Consistent with the results of Chapters 3 and 4, two pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$ appear to be more efficient for gene transfer by salmon sperm than one pulse of $1000 \text{ V/cm} \times 18.6 \text{ ms}$. Taken together with the findings presented in Chapter 3 and 4, these results demonstrate the repeatability of the salmon sperm electroporation technique, showing that it can be used for gene transfer into the embryos of more than one stock of chinook salmon. Additionally, it was also demonstrated that this technology is not limited to one species of salmon or one electroporation apparatus.

Southern analysis showed that OnMTGH1 DNA transferred by electroporated sperm was present in 0.88 day old (16 to 32 cells) and 5 day old (stage 9) chinook salmon embryos, with the equivalent of 40 pg (4.8×10^6 copies) detected per 5 day old embryo. This is comparable to the amounts of pRSV-lacZ DNA transferred by electroporated sperm into salmon embryos (Chapter 3). It appears that the DNA transferred into chinook salmon embryos by electroporated sperm and microinjection does not behave similarly. DNA introduced by microinjection was detected as high molecular weight DNA only,

with none of it in the original linear DNA form. In contrast to this, sperm transferred DNA was exclusively observed in the linear form. This was confirmed by the banding pattern obtained by restriction enzyme digestion of the DNA. These findings are in agreement with those outlined in Chapter 4, and support the conclusion that, unlike microinjected DNA which is converted into high molecular weight concatemers, sperm transferred DNA is unmodified after introduction into salmon embryos and remains as linear DNA molecules.

No evidence of the introduced growth hormone DNA was found in 10 month old chinook or coho salmon arising from sperm electroporated with this DNA. In addition, no obviously growth enhanced individuals were observed in these groups and none tested by PCR were found to contain OnMTGH1 DNA. In a normally distributed population of fish 95% and 99.7% of the population is expected to be within 2SD and 3SD of the mean (Tave, 1992). In some treatments the weight distribution did exceed the upper limit of these ranges. However, it is not uncommon for the weight distribution of groups of salmon to deviate from the pattern of this theoretical normal distribution. For example, even in a series of 40 full-sibling families from an inbred New Zealand chinook salmon stock, the proportion of individuals with weights greater than 2SD and 3SD above the mean was over 2.5% in 23 families and over 0.15% in 3 families, respectively (average weight, 10.3 to 20.2 g; number per group, 56 to 74) (Jane Symonds, personal communication).

It was not possible to compare between the weights of fish in different treatments due to the different rearing densities. Additionally, as the control groups perished in the hatchery, no comparisons can be made between treated and untreated fish from the same stock, reared under the same conditions. It was therefore not possible to determine if a particular sperm electroporation treatment influenced the average weight of the salmon in a particular group. However, it does not appear likely that this was the case. If the OnMTGH1 DNA was present at levels high enough, and in a large enough proportion of fish, to increase the average weight of a group, then it should have been detected by the PCR screening.

In contrast to the salmon resulting from electroporated sperm, microinjection of eggs with the GH gene construct did lead to fish significantly heavier than normal salmon of the same age. Most of these large fish were found to contain introduced DNA by PCR, indicating that growth enhancement was due to the presence of the OnMTGH1 construct. These findings are similar to those of other microinjection experiments carried out by Robert Devlin (personal communication). For example, in one study, where over 3000 eggs were injected, approximately 6% of the 12 month old coho salmon were transgenic in fin tissue, and most of these had significantly elevated growth rates (Devlin *et al.*, 1994). Comparable results were obtained when the opAFPGHc construct was introduced by egg microinjection into Atlantic, chinook, and coho salmon, and rainbow and cutthroat trout (Du *et al.*, 1992a; Devlin *et al.*, 1995). It should be noted that in these studies some fish containing the introduced GH construct did not demonstrate enhanced growth. This could be caused by several factors, such as the non-functioning of the gene, low copy number (mosaicism), or inappropriate expression due to position effect. This indicates that while phenotype allows rapid screening for transgenics, not all transgenic individuals will be detected by phenotypic analysis unless carried out in conjunction with DNA screening. Conversely, due to the mosaic nature of transgenics produced by egg microinjection, a fish may exhibit enhanced growth but may lack the introduced gene in some of its tissues. This was observed in this study in coho salmon, as 6 individuals (out of the 18 larger fish) had significantly enhanced growth rates but did not contain the introduced GH gene in their blood cells.

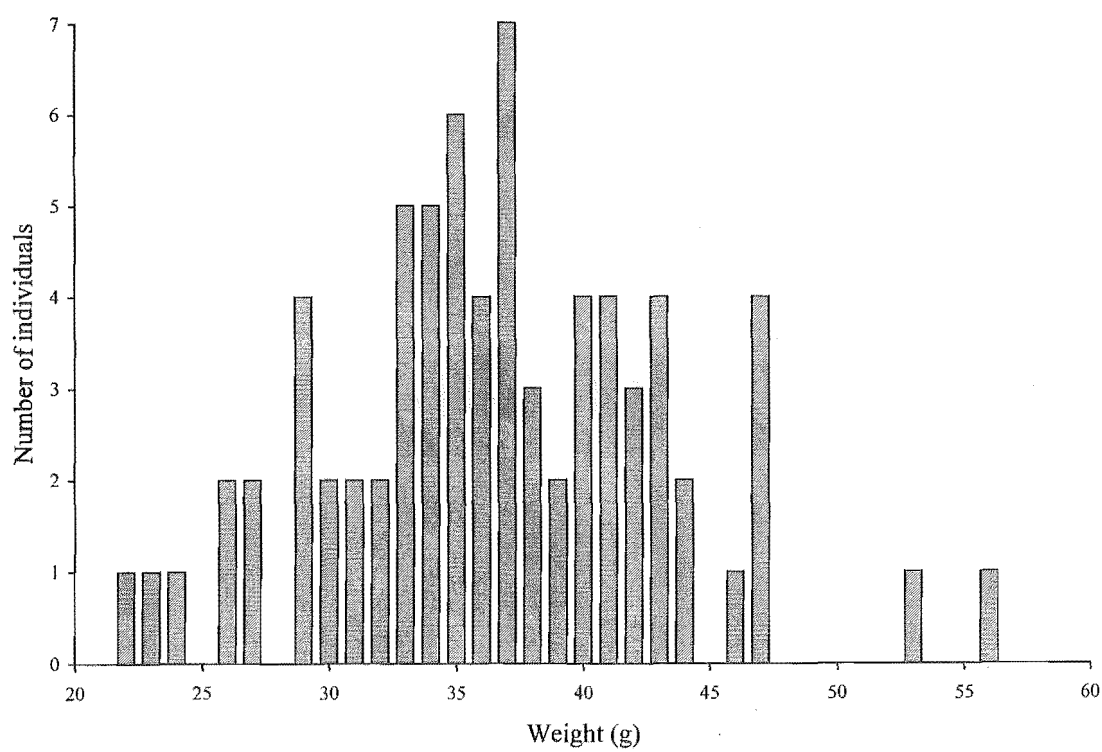
Unlike the results described in this Chapter, Tsai *et al.* (1995) reported the successful transfer of opAFPGHc DNA by electroporated loach sperm, and the production of growth enhanced fish. It is unclear why the electroporation of loach sperm was more successful than the current study in salmon. There may be many explanations for this disparity such as different sperm or seminal fluid characteristics of the two species, and/or the type of electric pulses delivered to the sperm. A noncapacitor Baekon instrument which delivers square electric pulses was used to treat the loach sperm. This delivered a different magnitude, number and duration, as well as a different type of pulse than the

capacitor based Cell Porator, which produces exponential decay pulses. To my knowledge electroporation of salmon sperm using a Baekon instrument has not been reported. This remains a possibility for future investigation, as while DNA is transferred efficiently into salmon embryos by sperm using exponential pulses, this DNA does not appear to persist within the developing fish.

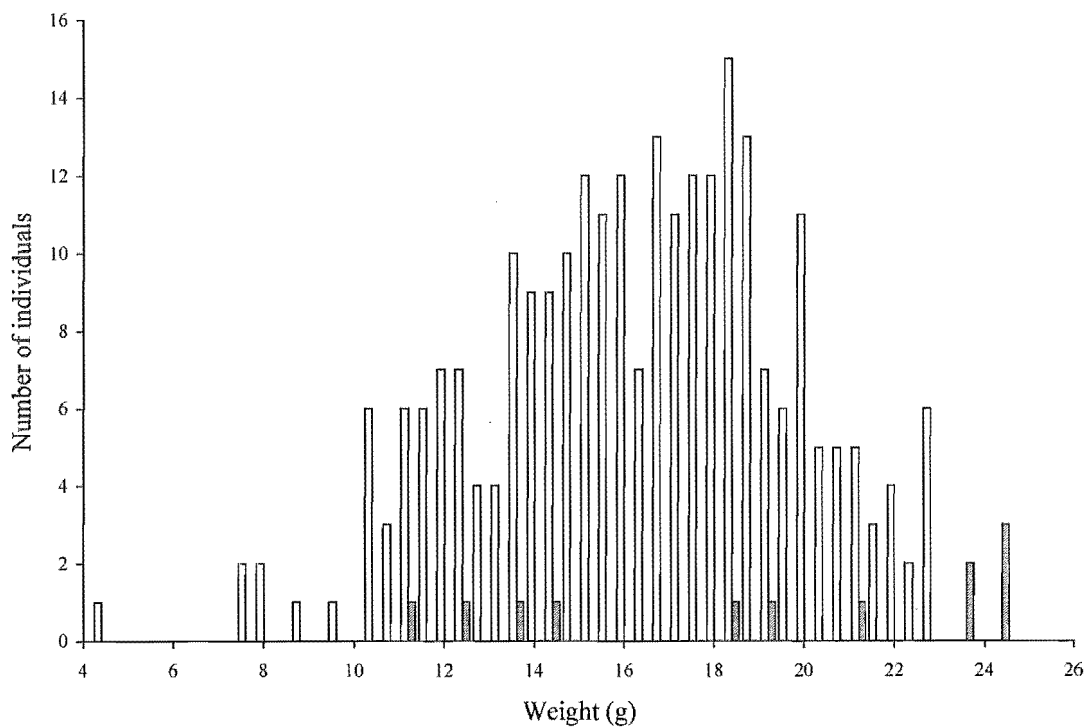
While electroporated salmon sperm are capable of transferring genes into salmon embryos, no growth enhanced transgenic fish were produced by this technique. Relatively high levels of introduced DNA were observed in embryos, but by 10 months post fertilisation no DNA was present, or was below the level of sensitivity of the detection method employed. The possibility remained that some highly mosaic transgenic individuals were produced which contained the DNA in tissues other than the fin, and did not exhibit the growth enhanced phenotype. However, if these fish did exist the chances of using them to produce a transgenic line are very low.

In conclusion, unless the DNA transferred by electroporated salmon sperm can be encouraged to persist and integrate at an early embryonic stage, then salmon sperm electroporation will not replace the egg microinjection methodology for the production of transgenic salmon. Possible ways of improving the sperm electroporation technique will be discussed in Chapter six.

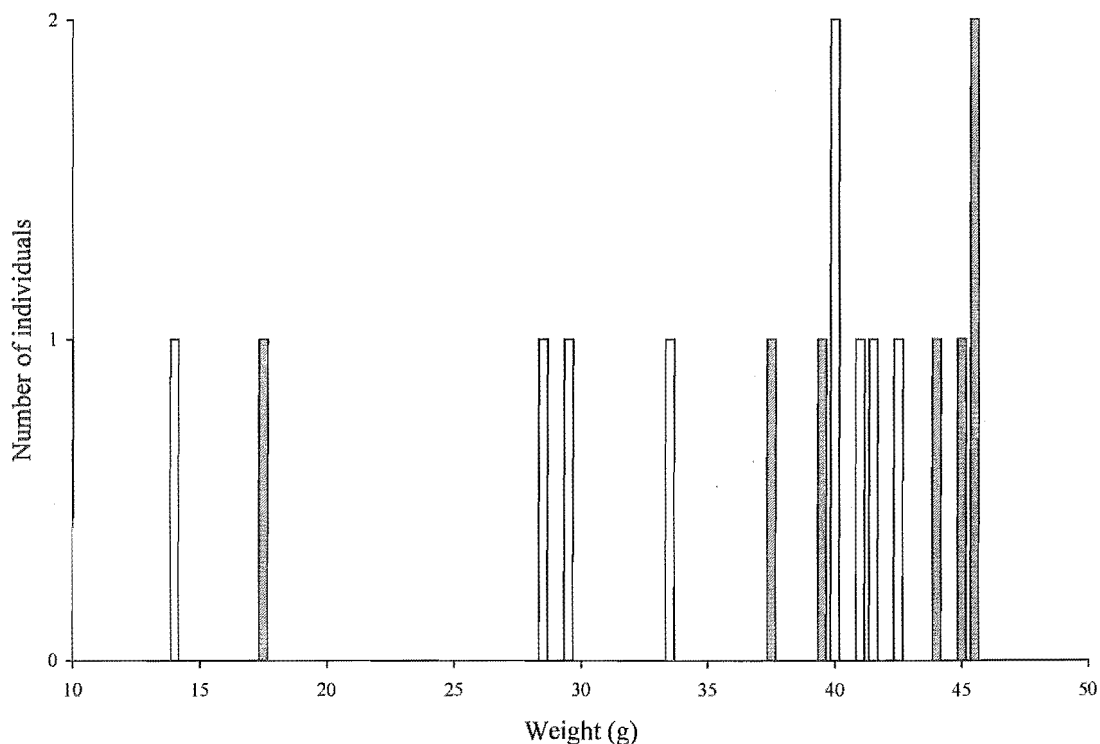
Figure 5.7. Frequency distribution of weights of ten month old salmon.
(a to e) Chinook salmon (treatments 8, 9, 10, 11 and 12) and (f to i) coho salmon (treatments 15, 16 18/19 and 21/22) resulting from sperm electroporated with OnMTGH1 DNA, and (j) coho salmon resulting from eggs microinjected with OnMTGH1 DNA. In the sperm electroporation treatments the individuals screened by PCR for the presence of introduced OnMTGH1 DNA in adipose fin tissue are indicated by the shaded bars. The weight of the individuals for which a blood sample was also analysed by PCR are indicated (B: treatments 11, 15 and 21/22).



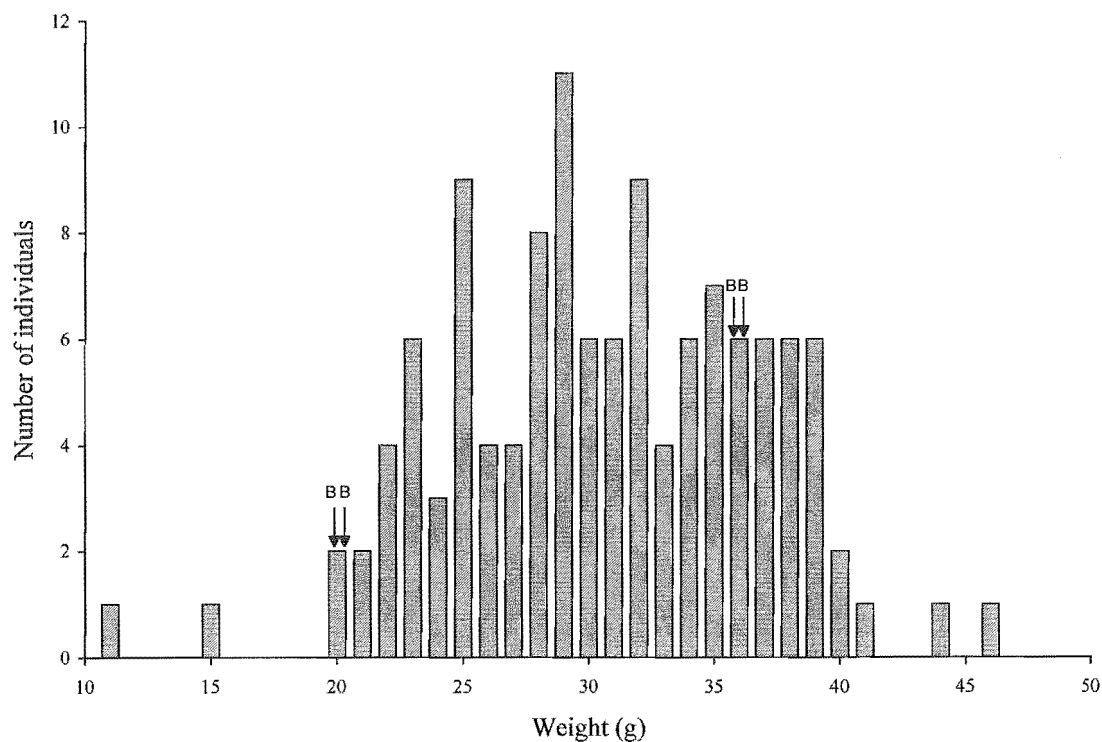
(a). Treatment 8. Chinook salmon resulting from sperm electroporated with 2 pulses of 1000 V/cm \times 27.4 ms with 200 μ g/ml of OnMTGH1 insert DNA. All of the fish in this treatment were screened for the presence of OnMTGH1 DNA by PCR.



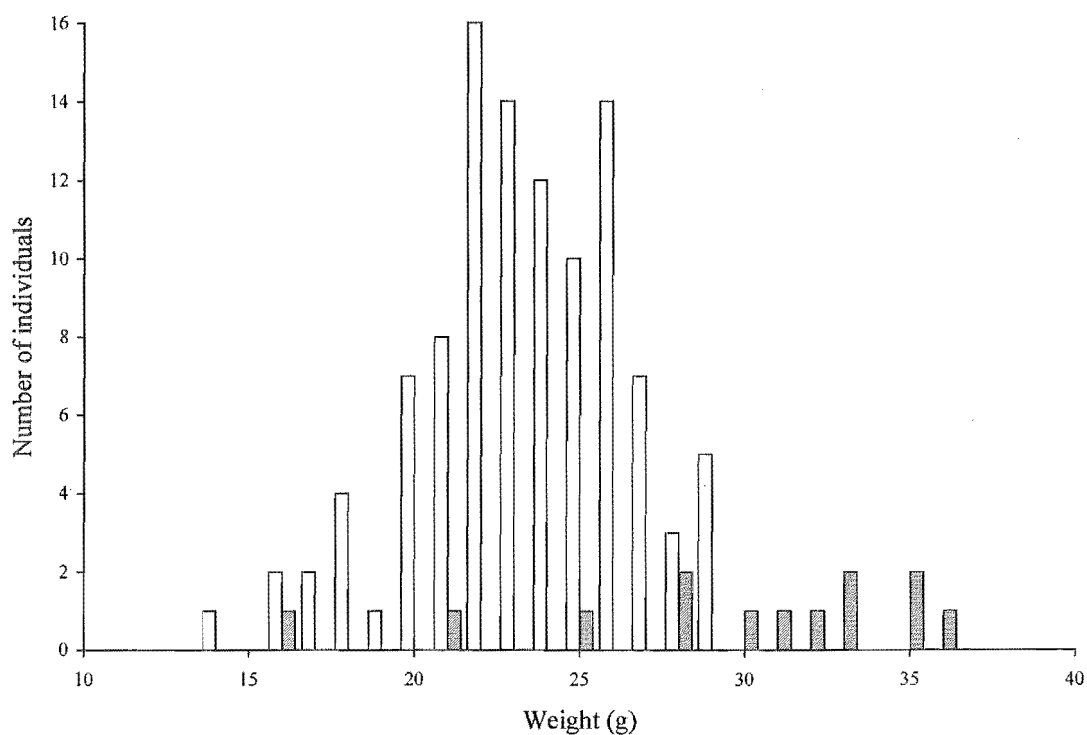
(b). Treatment 9. Chinook salmon resulting from sperm electroporated with 2 pulses of $1000\text{ V/cm} \times 27.4\text{ ms}$ with $20\text{ }\mu\text{g/ml}$ of OnMTGH1 insert DNA



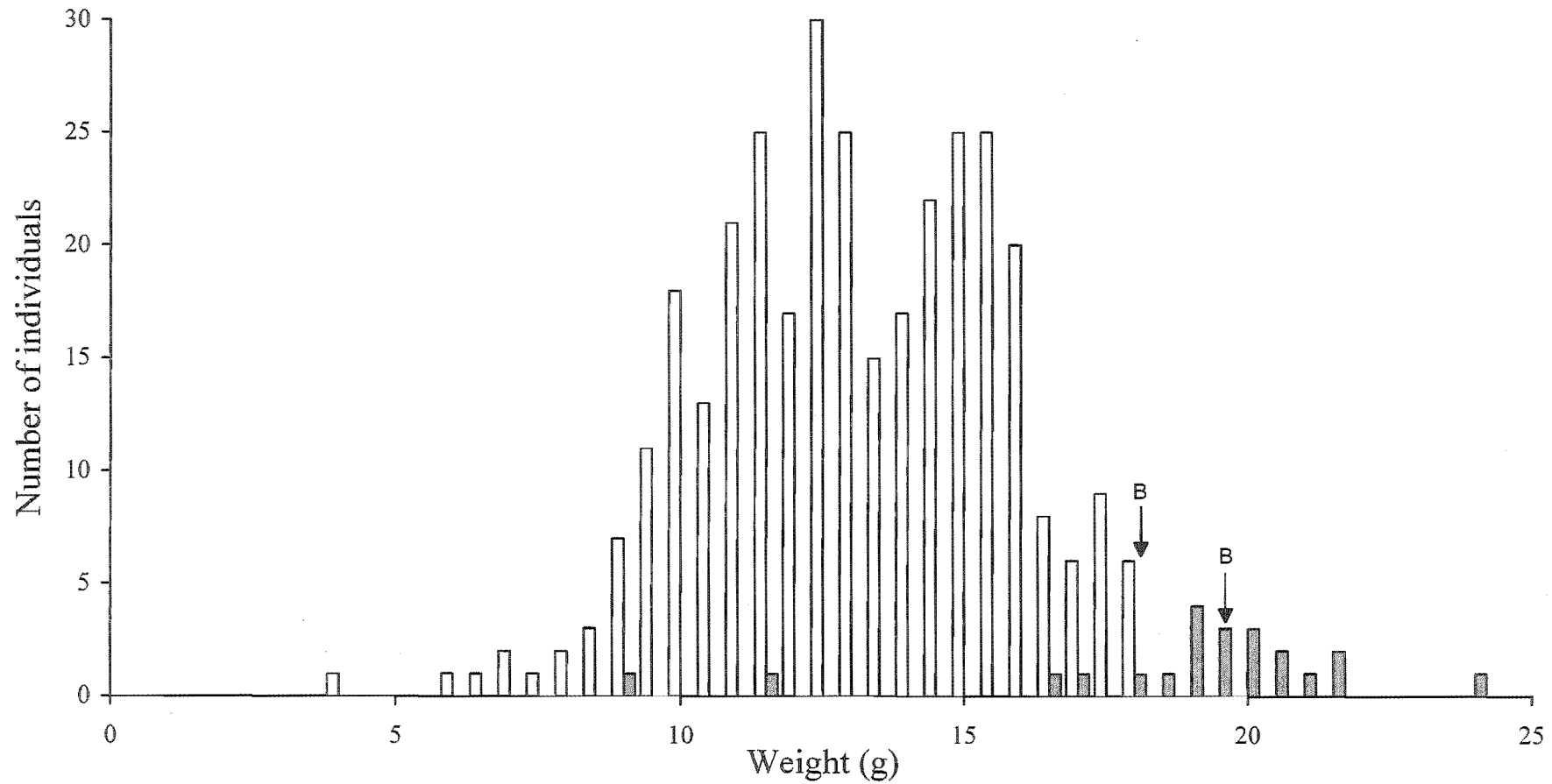
(c). Treatment 10. Chinook salmon resulting from sperm electroporated with 2 pulses of $1000\text{ V/cm} \times 27.4\text{ ms}$ with $20\text{ }\mu\text{g/ml}$ of OnMTGH1 insert DNA.



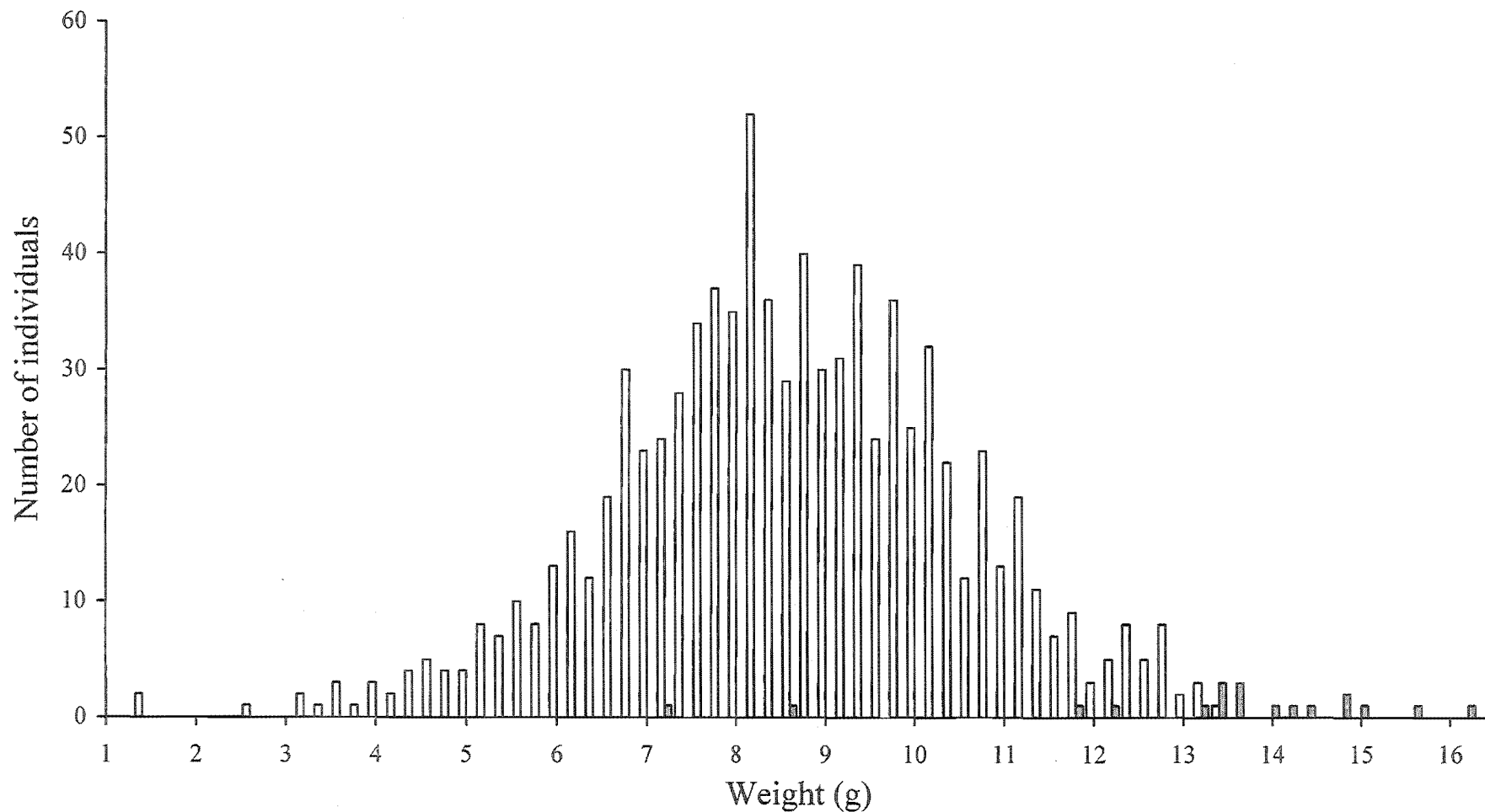
(d). **Treatment 11.** Chinook salmon resulting from sperm electroporated with 2 pulses of 1000 V/cm \times 27.4 ms with 200 μ g/ml of OnMTGH1 insert DNA.



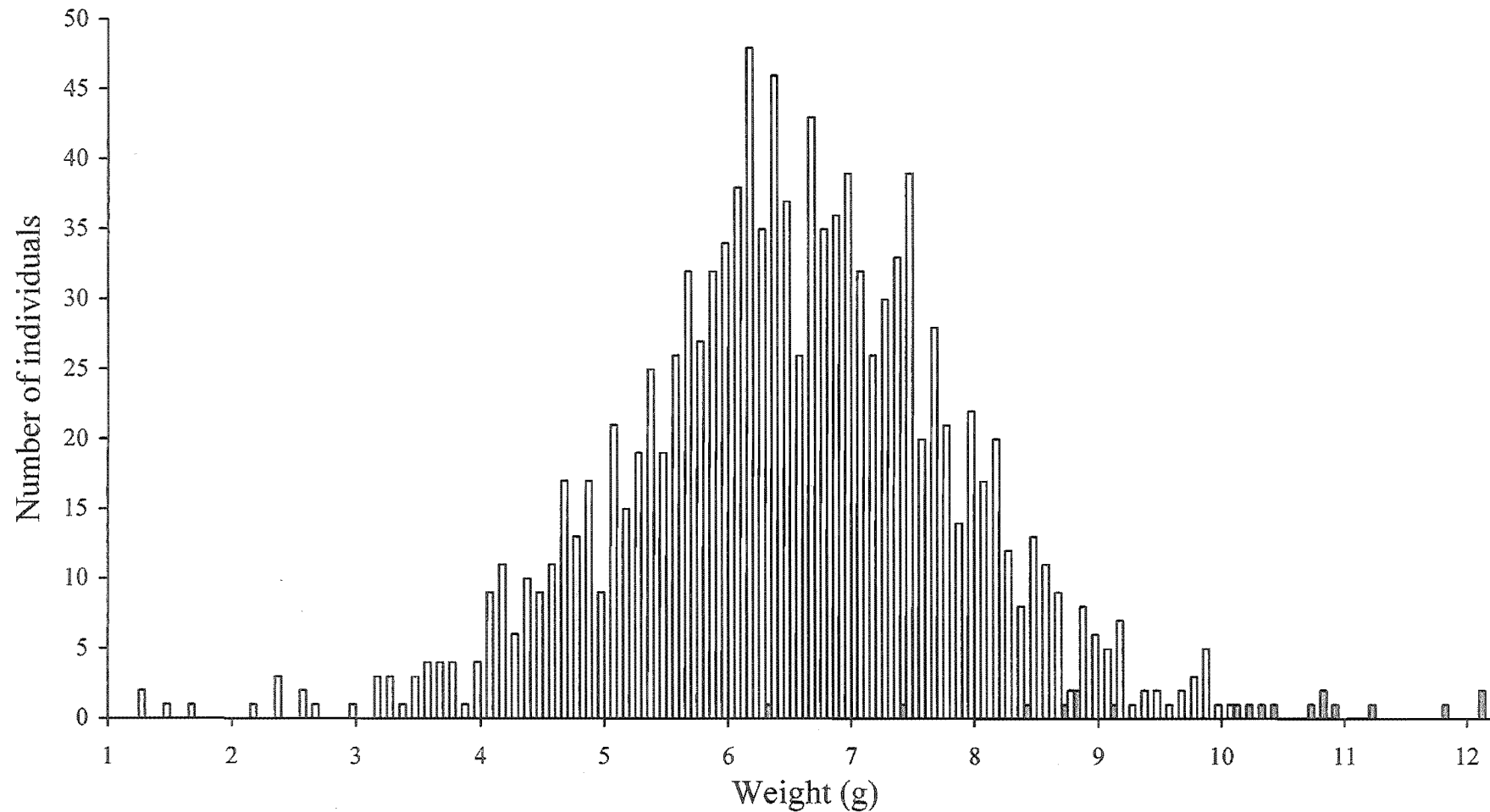
(e). **Treatment 12.** Chinook salmon resulting from sperm electroporated with 2 pulses of 1000 V/cm \times 27.4 ms with 200 μ g/ml of *NotI* linearised pOnMTGH1 DNA.



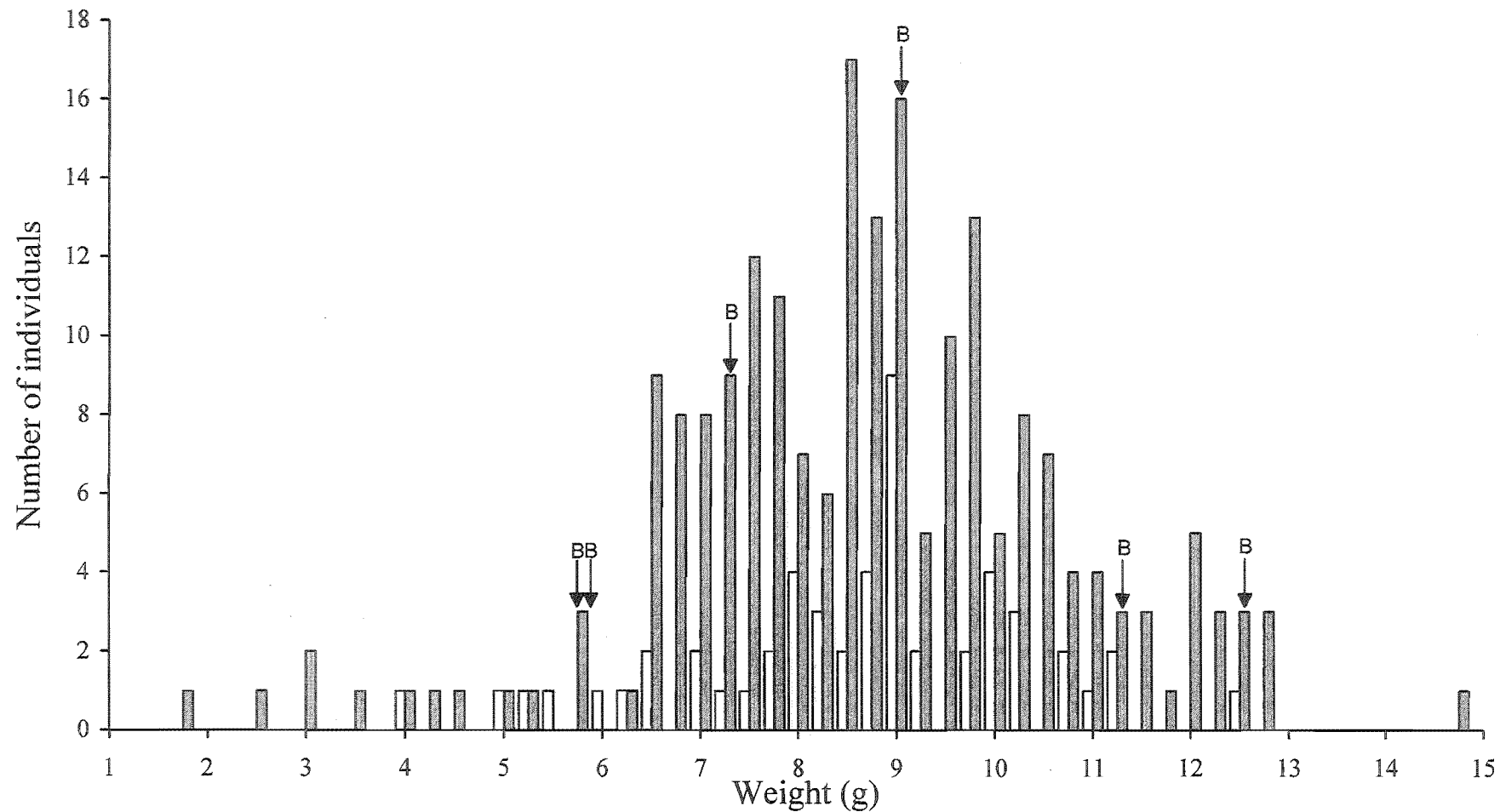
(f). Treatment 15. Coho salmon resulting from sperm electroporated with 2 pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$ with $200 \mu\text{g/ml}$ of OnMTGH1 insert DNA.



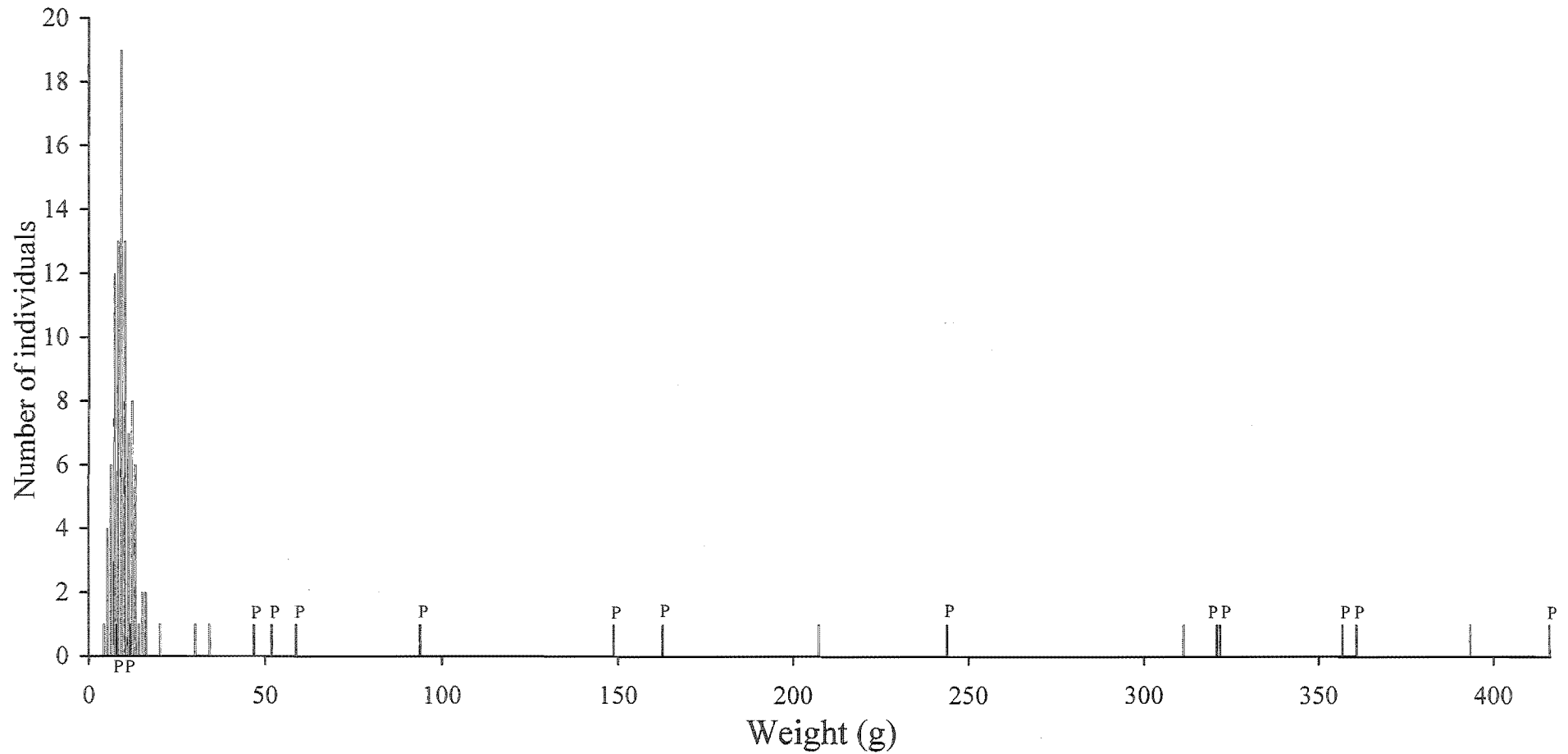
(g). **Treatment 16.** Coho salmon resulting from sperm electroporated with 2 pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$ with $200 \mu\text{g/ml}$ of OnMTGH1 insert DNA.



(h). Treatment 18/19. Coho salmon resulting from sperm electroporated with 2 pulses of 1000 V/cm \times 27.4 ms with 200 μ g/ml *Apa*I linearised pOnMTGH1 DNA.



(i). **Treatment 21/22.** Coho salmon resulting from sperm electroporated with 2 pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$ with $200 \mu\text{g/ml}$ of *NotI* linearised pOnMTGH1 DNA.



(j). **Microinjection treatment.** Coho salmon resulting from eggs microinjected with OnMTGH1 DNA. Blood samples from all individuals were screened for the presence of introduced OnMTGH1 DNA by PCR. Those found to be positive (P) are indicated by the black bars.

Chapter Six.

General discussion.

The main aim of this thesis was to evaluate the use of sperm for gene transfer in chinook salmon, and to determine if transfer efficiency was enhanced by electroporation of the sperm. Additional aims included optimising the conditions for gene transfer using electroporated sperm and determining the fate and expression of the sperm transferred DNA. I also wanted to compare the technique of sperm electroporation with egg microinjection and determine if growth enhanced fish could be produced after salmon sperm were electroporated with plasmid DNA containing a salmon growth hormone gene.

The results presented show that although DNA is taken up from solution by salmon sperm during simple incubation, electroporation of the sperm/DNA mixture significantly enhances DNA uptake. The amount of introduced DNA detected in embryos and the proportion of individuals containing this DNA was also greater when electroporated sperm were used for gene transfer. This is consistent with other studies examining the effect of electroporation on gene transfer by fish sperm (Müller *et al.*, 1992; Sin *et al.*, 1993; Tsai *et al.*, 1995; Patil and Khoo, 1996).

Several factors were identified as being important in determining the amount of DNA taken up by the sperm and the efficiency of gene transfer using electroporated sperm. In particular, the field strength, pulse length, number of pulses and DNA concentration were shown to significantly affect the uptake and transfer. The effect of the concentration of the buffer, the time the sperm was incubated on ice after electroporation, the form of the DNA (circular and linear) and the inclusion of carrier DNA, was also evaluated. Optimal conditions were found to be two pulses of 27.4 ms at 1000 V/cm, with 200 µg/ml of plasmid DNA in 0.5 × HBS, a ten minute incubation on ice after electroporation and without carrier DNA. The form of the DNA was not found to significantly affect the transfer efficiency by electroporated salmon sperm. Overall, electroporation of sperm was shown to be an effective method for transferring genes into salmon embryos. Efficient transfer was consistently achieved over four breeding seasons,

in two species of salmon, and with two electroporators. Additionally, similar gene transfer results were obtained using three different gene constructs.

Examination of the fate of pRSV-lacZ and pSV2CAT DNA transferred into salmon embryos by electroporated sperm (Chapter 4) revealed that by the morula stage (1.2 days old) relatively high amounts of DNA were present (up to 1×10^7 copies of the plasmid). When circular plasmid DNA (in the form of supercoiled and relaxed open circular DNA) was introduced by the sperm it was detected predominantly as covalently closed and relaxed open circular DNA. When embryos developing from sperm electroporated with linear plasmid DNA were analysed the introduced DNA was mainly detected in the linear form. No evidence was obtained for amplification of the introduced pRSV-lacZ and pSV2CAT DNA after the morula stage, and only decreasing levels were observed as the fish developed. Analysis of embryos into which linear OnMTGH1 DNA had been introduced, confirmed that introduced DNA remains unmodified and in the linear form (Chapter 5). Therefore, it appears that the DNA introduced into salmon embryos by sperm is amplified during the early stages of embryo development, but remains extrachromosomal in an unmodified form. The replication of the sperm transferred DNA in salmon embryos must have occurred prior to the 16 to 32 cell stage as relatively high levels were observed in samples of this stage (at least 2×10^6 copies). In contrast to the sperm transferred DNA, all the OnMTGH1 DNA introduced into salmon by egg microinjection had been transformed into higher molecular weight forms by the 16 to 32 cell stage (Chapter 5).

Contrary to my findings in salmon, Patil (1996), found that some linear and circular plasmid DNA introduced into zebrafish embryos by sperm electroporation, was converted into high molecular weight concatemers. Additionally, the linear plasmid DNA introduced into the zebrafish by electroporated sperm was rapidly re-circularised, as the plasmid DNA was detected as supercoiled circular DNA in one hour old embryos, while none was detected as linear molecules. DNA introduced by microinjection into the zebrafish embryos was amplified during development with a peak accumulation in late gastrulation, 10 hours post-fertilisation. Results for DNA transferred by electroporated

sperm were similar, except the levels of introduced DNA present in the embryos were much less than following introduction by microinjection. However, Patil (1996) found that the DNA introduced by microinjection was mostly replicated as high molecular weight concatemers, whereas the majority of the sperm transferred DNA was amplified in the supercoiled circular form, and only a small proportion as high molecular weight DNA. Therefore, it appears that plasmid DNA introduced into salmon and zebrafish by electroporated sperm is less likely to be converted into high molecular weight concatemers during embryogenesis than microinjected DNA.

Since high levels of introduced linear DNA were detected in salmon embryos following sperm transfer it appears that the linear plasmid DNA is replicated in the linear form during embryogenesis. This is not consistent with observations of DNA introduced by microinjection into *Xenopus* (Rusconi and Schafner, 1981; Marini *et al.*, 1988; Endean and Smithies, 1989; Asano and Shiokawa, 1993), sea urchin (McMahon *et al.*, 1985), zebrafish (Stuart *et al.*, 1988), and medaka (Chong and Vielkind, 1989), where introduced linear DNA was converted into concatemers before being replicated, and no increase in the amount of linear DNA was observed. From these studies it appears that the transformation of linear plasmid DNA into higher molecular weight concatemers is a prerequisite for replication. It has also been suggested that there may be a minimum size requirement for DNA which can initiate the formation of nucleus like structures necessary for DNA replication (Marini and Benbow, 1991). In zebrafish, unlike salmon, the DNA transferred by sperm does not persist in the linear form (Patil, 1996). Due to observations of the delayed replication of injected linear DNA relative to circular molecules, and as the replication of linear molecules was inhibited by dephosphorylation, Patil (1996) concluded that linear DNA must first be re-circularised before it can be replicated. If this is the case, then it is difficult to explain how the linear DNA in salmon embryos was replicated to the levels observed following sperm transfer without re-circularisation. As previously discussed in Chapter 2, when salmon sperm are electroporated in the presence of 100 µg/ml of plasmid (pRSV-lacZ) DNA, a maximum of 1.5 fg (200 copies) of the DNA appears to be strongly associated on average with each sperm (including DNase sensitive

DNA). Therefore this cannot account for the high levels of DNA detected in salmon embryos (greater than 100 pg), and replication of the DNA following transfer appears more likely than the introduction of high levels of DNA during fertilisation. However, the exact mechanism of how the DNA is replicated in the linear form is not clearly understood.

Although the DNA transferred into salmon by electroporated sperm was detected in embryos, with up to 2×10^6 , 1×10^7 , 5×10^6 , and 1×10^5 copies observed in 0.8, 1.2, 5 and 11 day old embryos respectively, the introduced DNA did not persist in the salmon. Southern analysis of embryo DNA showed that decreasing amounts of introduced pRSV-lacZ and pSV2CAT DNA were present in progressively older individuals (Chapters 3 and 4). The amount of introduced DNA present in twelve week old fry was below the level of detection of Southern analysis. Similar results were also obtained when the growth hormone gene construct, OnMTGH1, was introduced into chinook and coho salmon by electroporated sperm (Chapter 5). Embryos, 0.8 and 11 days post-fertilisation, were found to contain introduced DNA, but no growth enhanced individuals were obtained and no OnMTGH1 DNA was detected in 10 month old fish. In contrast, when this construct was transferred into coho salmon by egg microinjection, the DNA was found to persist in 10 month old fish and growth enhanced individuals were obtained. In addition, no evidence of integration of DNA transferred by salmon sperm was obtained. However, the possibility cannot be excluded that a portion of the DNA was integrated in some cells, but was at a low level due to the highly mosaic nature of the fish.

Other studies examining sperm mediated gene transfer have observed mosaicism and limited persistence of introduced DNA. For example, sperm transferred DNA was detected in mice embryos from the 1 cell to the hatched blastocyst stage, but no evidence of gene transfer was observed in any of the newborn mice analysed (Hochi *et al.*, 1990). The successful production of transgenic adult pigs using this method was reported by Gandolfi *et al.* (1989), with 10 out of 48 individuals tested containing the transgene. However, the introduced gene was not transmitted to the F1 progeny, indicating that highly mosaic animals are produced by this technique (Lauria and Gandolfi, 1993). It

seems that integration of the introduced DNA is unstable, and the DNA is either lost during development, or highly mosaic animals are generated (Lauria and Gandolfi, 1993).

Results from more recent studies indicate that sperm transferred DNA does persist in fish under some conditions. In a study carried out in 1995, involving gene transfer by electroporated chinook salmon sperm, evidence was obtained by PCR analysis that the introduced DNA was still present in 15 month old individuals (3 out of 16 individuals were found to contain the introduced DNA, Frank Sin, personal communication). Additionally, Southern analysis of restriction enzyme digested DNA extracted from the blood of one individual indicated that more than one copy of the introduced gene was present per cell, and showed a banding pattern consistent with integration of the introduced DNA. In the 1995 study a construct consisting of the chinook salmon metallothionein promoter with a coho salmon IGF-I cDNA clone and SV40 polyadenylation signal was transferred, and the electroporation conditions used were those optimised in this thesis (two pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$, with $100 \mu\text{g/ml}$ of DNA). The reasons for the apparent greater persistence of the sperm transferred DNA in this study, relative to the results of my thesis, are unclear. Transfer may have been more efficient in the 1995 breeding season due to individual differences in the sperm, or alternatively to differences in the plasmids used.

Persistence and germline transmission of DNA transferred into zebrafish by sperm has also recently been demonstrated by Patil (1996). The DNA introduced by sperm incubation and electroporated sperm was observed in adult fish and was transferred to subsequent generations. However, the pattern of inheritance in the F2 and F3 generations was consistent with the passage of extrachromosomal DNA and was not consistent with integration of the introduced DNA.

In Chapter 4, successful expression of the DNA introduced by electroporated sperm was demonstrated in embryos. However, expression was detected in only a small percentage of the embryos despite the presence of high levels of plasmid DNA in most of the embryos analysed. In the DNA uptake study (Chapter 2) as well as gene transfer studies (Chapters 3 to 5) there was some evidence to suggest that the DNA was altered or damaged in some way after electroporation with two pulses of $1000 \text{ V/cm} \times 27.4 \text{ ms}$. For

example, PCR failed to detect plasmid sequences in DNA extracted from sperm following electroporation under these conditions (20 µg/ml treatment), despite DNA being detected using less sensitive hybridisation techniques (Chapter 2). Similarly, in Chapter 4, a small proportion of the introduced DNA was detected as novel sized bands in 4 of the 46 positive embryo DNA samples analysed. All 4 samples were from embryos resulting from sperm treated with this two pulse treatment, indicating that the introduced DNA had undergone some form of alteration in these embryos. In three of these samples the novel sized bands had a higher molecular weight than unmodified plasmid, and could represent concatemerisation of the plasmid DNA or other rearrangements post electroporation. However, the possibility remains that the novel bands were caused by the electroporation procedure.

It is possible that alterations to the DNA are responsible for the low incidence of expression of the plasmid DNA introduced by electroporated sperm. However, despite the low frequency, it was nevertheless demonstrated that expression can be gained following the exposure of the DNA to an electric field. For example, expression of pRSV-lacZ was detected by ONPG analysis in both the one pulse, 1000 V/cm \times 18.6 ms, and two pulse, 1000 V/cm \times 27.4 ms, treatments. Additionally, as HB101 transformants were obtained from sperm DNA following electroporation with the harshest conditions, the ampicillin resistance gene was shown to remain functional in at least a portion of the plasmid DNA after such treatment. Other studies have observed damage to cellular DNA by electroporation. A study by Vatteroni *et al.* (1993) indicated that electroporation induces an increase in chromosomal aberrations, sister-chromatid exchanges and deletions in the DNA of Chinese hamster cells. It has also been demonstrated that a portion of the cell death caused by electroporation is due to double stranded breaks in the cellular DNA and that the number of such breaks increases with increased field strength and pulse length (Meaking *et al.*, 1995). If the DNA taken up by electroporated salmon sperm is being damaged by the electric field, only minor changes must be induced in the plasmid DNA, as no large rearrangements were detected by Southern analysis. There was no evidence of damage of the DNA transferred by electroporated fish sperm in any other studies (Müller *et al.*, 1992; Sin *et al.*, 1993; Tsai *et al.*, 1995; Patil, 1996), and successful expression was

obtained from DNA transferred by electroporated sperm into African catfish (Müller *et al.*, 1992).

As discussed, only limited expression of pRSV-lacZ and pSV2CAT was observed in salmon embryos, and no expression of the OnMTGH1 gene construct was evident following transfer. Similarly, no evidence of expression was detected from several plasmids transferred into zebrafish embryos by electroporated sperm, while expression was observed when egg microinjection was employed (Patil, 1996). In salmon and zebrafish, plasmid DNA was less likely to be transformed into concatemers following transfer by electroporated sperm than when transferred by microinjection. A study by Krebs and Dunaway (1996) examining the expression of genes injected into *Xenopus* oocytes indicated that the level of eukaryotic transcription is related to the length of the DNA template. In their study increasing amounts of transcription were achieved from progressively longer DNA molecules and evidence of a minimum length required for transcription of between 14 and 17 kb was obtained. Such a length requirement may explain the low frequency of expression of sperm transferred DNA in salmon, as the DNA remains as discrete plasmid monomers less than 8 kb in length.

The lack of persistence and decreasing DNA levels observed following DNA transfer into salmon by sperm may also be related to the lack of concatemerisation of the plasmid DNA. Concatemers of plasmid DNA may be more stable and less likely to be degraded than plasmid monomers. Additionally, the concatenation of the introduced DNA may increase the likelihood of its integration into the genome. The DNA injected into fish embryos is rapidly transformed into concatemers (see Chapter 4 and 5) and transgenes are often observed to be integrated as tandem arrays of multiple copies in fish (Chen *et al.*, 1995; Iyengar *et al.*, 1996). The analysis of integration events following gene transfer in mice has also indicated that introduced DNA molecules first form concatemers prior to their integration (Hamada *et al.*, 1993). Alternatively, the lack of expression and persistence of DNA introduced into salmon by electroporated sperm may be due to inappropriate partitioning of the DNA in the embryonic cells. This may prevent the plasmid DNA coming into contact with the enzymes and other cellular components necessary for expression, concatenation and integration.

The use of sperm electroporation for gene transfer into salmon compares favourably to microinjection. Up to 85% of salmon fry developing from electroporated sperm were found to contain introduced DNA (Chapter 3) while less than 10% of salmon resulting from microinjected eggs are reported to retain the foreign DNA (Fletcher *et al.*, 1988; Shears *et al.*, 1992; Devlin *et al.*, 1994; Devlin *et al.*, 1995). However, it is difficult to compare directly between these studies as different developmental stages were examined. When sperm electroporation and egg microinjection were directly compared by the transfer of OnMTGH1 DNA, microinjection was found to be more efficient for gene transfer into 10 month old salmon (Chapter 5). The introduced DNA was detected in 12% of the salmon produced by microinjection and growth enhanced individuals were detected. In comparison neither introduced DNA or growth enhancement was evident in 10 month old salmon developing from sperm electroporated with the GH construct. This is most likely due to the reduced persistence of the DNA introduced by sperm rather than a lower efficiency of introducing DNA into embryos, as a greater proportion of 11 day old embryos resulting from sperm electroporation (5/5) were found to contain OnMTGH1 DNA than those resulting from microinjection (4/8). Patil (1996) also directly compared sperm electroporation with microinjection techniques for gene transfer into zebrafish. Lower levels of introduced DNA were observed in embryos developing from electroporated sperm. However, the introduced DNA was found to persist in a similar proportion of adult zebrafish resulting from sperm electroporation (16 to 26%) and egg microinjection (13 to 25%). Therefore, Patil (1996) concluded that sperm electroporation was more efficient as a gene transfer technique as it could be applied to a greater number of eggs.

While electroporated sperm have been shown to be effective vectors for transferring DNA into salmon embryos the introduced DNA does not persist and the amount decreases as the fish develop. Nevertheless, given the ease with which electroporated salmon sperm can be used to transfer genes into a large number of embryos, further work on this method is justified.

6.1 Opportunities for future work.

In this study evidence was gained that plasmid DNA is taken up from solution and internalised by electroporated chinook salmon sperm. Firstly, the plasmid DNA became strongly associated with the sperm, and resisted multiple washes and digestion with DNase, and secondly the DNA was transferred into embryos upon fertilisation. In a similar study, Patil (1996) showed by ultrastructural *in situ* hybridisation followed by autoradiography of the sperm, that a portion of the plasmid DNA was taken up by electroporated zebrafish sperm and internalised into the sperm nuclei. It would therefore be of interest to apply similar methods to salmon sperm after electroporation with DNA and determine the site of internalisation. Such methodology may lead to increased efficiency of this gene transfer method if the amount and site of internalisation by the sperm could be related to the conditions of the electroporation and the efficiency of transfer into embryos, in terms of the amount of DNA transferred, the number of transgenic individuals and the fate of the introduced DNA.

Although electroporated sperm transfer DNA into a large proportion of embryos, the incidence of expression is low and the DNA does not persist. Therefore to optimise this technique further it is necessary to understand what happens to the sperm transferred DNA post-fertilisation. For example, the inability of the DNA to be expressed efficiently may be due to inappropriate partitioning of the introduced DNA in the embryonic cells. Therefore, it would be worthwhile to determine the sequence of events following fertilisation. Methods such as the transfer of labelled DNA, or *in situ* hybridisation to introduced DNA, followed by autoradiography could be applied to determine the location of the introduced DNA in embryos. Examination of developmental stages prior to the morula and 16 to 32 cells stages would provide information about the replication of the sperm transferred DNA in salmon. Further understanding of the fate of the introduced DNA may lead to strategies to increase the efficacy of this technique. For example, if the majority of the DNA is not reaching the nucleus then techniques that target the passage to this organelle could be evaluated. However, if significant amounts of introduced DNA were shown to exist in the nucleus then methods that increase the integration frequency of introduced DNA may be more appropriate.

The salmon sperm electroporation technique used in this study involved only the introduction of naked plasmid DNA and did not investigate the possibility of co-transferring factors that may facilitate the production of transgenics. There are several approaches that could be applied in conjunction with sperm electroporation to encourage the persistence and/or integration of DNA introduced by this method. The association of the transferred DNA with molecules that prolong the length of time the DNA survives in the cellular environment may increase the chance of it becoming associated with the nuclear DNA. For example, the binding of plasmid DNA to the core protein of adenovirus type 2 and to salmon sperm protamine has been shown to increase the efficiency of transport of the protein-DNA complex into cell nuclei (Wienhues *et al.*, 1987).

Page *et al.* (1995) found that cytoplasmic injection of DNA, bound in a condensed form with poly-L-lysine, into pronuclear stage mouse embryos resulted in a gene transfer efficiency of 12.8%. In comparison when the same amount of naked DNA was injected into the cytoplasm no transgenic mice resulted. Presumably the presence of poly-L-lysine with the plasmid DNA offers protection from degradation processes, such as the action of cellular nucleases, and may encourage nuclear localisation and integration by other mechanisms (Page *et al.*, 1995).

The binding of plasmid DNA to SV40 T antigen nuclear localisation sequence (NLS) peptide prior to cytoplasmic injection into 1-2 cell zebrafish embryos resulted in efficient targeting of the DNA to the nucleus (Collas *et al.*, 1996). Higher rates of expression from the introduced DNA were obtained when the DNA was bound to the NLS and expression was observed in 35% of embryos when as few as 10^3 copies were injected into the cytoplasm, whereas no evidence of expression was observed when the NLS was not included.

Another strategy that has been attempted to increase the integration of introduced DNA, is the use of enzymes that affect the interaction of the introduced DNA with the host chromosomal DNA. For example, viral integrase proteins are known to cleave chromosomal DNA and mediate integration between viral cDNA and the host chromosome (Ivics *et al.*, 1993). When viral integrase proteins and a DNA vector containing a gene flanked by integrase recognition sequences were co-injected into

zebrafish embryos, increased integration was indicated by increased persistence of the introduced DNA, higher expression and evidence from ligation mediated PCR (Ivics *et al.*, 1993).

Another approach is the co-transfer of restriction endonucleases with plasmid DNA. Schiestl and Peters (1991) found that the efficiency of transformation of yeast cells was increased by the co-transfer of the restriction enzyme *Bam*HI and plasmid DNA cut with the same enzyme. Additionally, the introduced DNA was often integrated into *Bam*HI sites of the yeast genome. Such restriction enzyme-mediated integration (REMI) has been successfully used for insertional mutagenesis in the slime mold *Dictyostelium* (Kuspa and Loomis, 1992). When restriction enzyme was included, the rate of transformation was increased by over 20-fold. Additionally, the introduced DNA was integrated at sites determined by the restriction enzyme in greater than 70% of the transformants. When the co-injection of zebrafish embryos with restriction enzyme and plasmid DNA was carried out, some evidence for increased integration was obtained relative to the injection of plasmid alone (Catanach, 1995). In this study putative junction fragments detected by IPCR were detected in a greater number of individuals when the restriction enzyme was included. REMI has also been used in the production of transgenic *Xenopus* (Kroll and Amaya, 1996). In this study the restriction enzyme and plasmid DNA were incubated with decondensed sperm nuclei prior to their transplantation into eggs. The inclusion of the restriction enzyme in this procedure enhanced the frequency with which non-mosaic expression from the introduced DNA was obtained. Given these results, it is of interest to determine if combining a similar REMI method with the salmon sperm electroporation procedure can result in increased persistence of DNA introduced by this method, or integration of the DNA into the salmon genome at an early developmental stage.

The chromosomal DNA contained in sperm is in a highly condensed form bound to protamines (Dadoune, 1995). A recent study has attempted to increase the efficiency of gene transfer by electroporated chinook sperm salmon by transferring protamine bound DNA (Frank Sin, personal communication). It is hoped that the condensed protamine bound DNA will be protected from degradation and may follow the normal course of

DNA originating from sperm and become internalised into the nucleus. Preliminary results from this study suggest that the protamine bound introduced DNA is more likely to be retained than the naked plasmid DNA (transfer conditions were two pulses of 1000 V/cm \times 27.4 ms, with 100 μ g/ml of DNA). A larger proportion of 15 month old salmon were found to contain the introduced DNA in their fin tissue when protamine was used (5/15) than in the group originating from sperm electroporated with naked DNA (3/16), and all fish in the protamine treated group were found to contain introduced DNA in at least one tissue (Frank Sin, personal communication).

Due to the supposed role of topoisomerase I in illegitimate recombination, through the cleavage and religation of nonhomologous ends (Chen *et al.*, 1996; Zhu and Schiestl, 1996; Wang, 1996), plus evidence that transgenes in mice integrate near topoisomerase I cleavage sites (Hamada *et al.*, 1993; Chen *et al.*, 1996), this enzyme may also be of use in encouraging the integration of introduced DNA. The inclusion of this enzyme has recently been shown to have a positive effect on the sperm gene transfer procedure in zebrafish (Walker, 1996). The addition of topoisomerase I increased the number of 5 day old fry found to contain introduced DNA from 0 to 50% (12/24) when sperm incubation was used, and from 37% (11/30) to 60% (29/48) when electroporated sperm were used. Additionally, the introduced DNA was only detected in four month old zebrafish resulting from sperm electroporated in the presence of plasmid DNA and topoisomerase I. Although there was no direct evidence that the introduced DNA was integrated into the genome of these fish, transmission of the introduced DNA to the next generation was demonstrated. It therefore appears that topoisomerase I increases the retention of the DNA as well as the efficiency of gene transfer by electroporated zebrafish sperm.

Other approaches for improving gene transfer methodology in fish concern the development of more efficient vectors that themselves mediate integration of the gene of interest. Transposons are mobile genetic elements that mediate integration by the integrase enzymes they encode, and have been used to introduce genes into the germline of *Drosophila* (Spradling and Rubin, 1982). Tc1-like putative transposable elements have been identified in zebrafish (Izváková *et al.*, 1995) and Atlantic salmon (Goodier and

Davidson, 1994). The reconstitution of a functional transposon for use as a transgene vector in fish may be possible in the near future (Izváková *et al.*, 1995).

Retroviral vectors have been used to transfer genes into cultured cells (Eglitis and Anderson, 1988; Mehigh *et al.*, 1993), tissues (Archer *et al.*, 1994), cattle embryos (Kim, 1993), and the germ line of mice (Jahner *et al.*, 1985; Jaenisch, 1988) and chickens (Bosselman *et al.*, 1988). This method has several positive aspects including the ease of infecting cells in a wide range of developmental stages, the ability to isolate the flanking regions of the integration site, and the integration of a single copy of the DNA into the host genome via the action of the viral integrase (Jaenisch, 1988). However, the size of the DNA that can be introduced is limited to approximately 8 kb and inefficient expression often results from DNA introduced via viral vectors (Eglitis and Anderson, 1988; Jaenisch, 1988). Gene transfer into zebrafish (Lin *et al.*, 1994; Gaiano, *et al.*, 1996) and medaka (Chen *et al.*, 1995) has been successfully carried out using a retroviral vector. However, the rate of production of transgenic fish using this technique has been limited by the use of microinjection to introduce the viral particles. By combining this technique with sperm electroporation these limitations may be overcome.

The use of retroviral vectors in the application of gene transfer to aquaculture may be limited. Due to negative public perceptions, regulations may exclude the inclusion of vector sequences, both bacterial or viral, and may only allow sequences from closely related species to be used (Hackett, 1993; Gong and Hew, 1995). Additionally, safety requirements may also mean that the potentially infectious vector has to be adequately rendered non-replicating, and innocuous (Berkowitz and Kryspin-Sørensen, 1994).

Another method that may become available in the future for the more efficient and accurate production of transgenic salmon is the use of embryonic stem (ES) cells. These pluripotent cells, which can be manipulated in culture and then introduced into embryos, have been used successfully for introducing DNA into the germline of mice (Gossler *et al.*, 1986) and in gene targeting experiments in mice (Thomas *et al.*, 1986; Thomas and Capecchi, 1987). ES cell technology is being developed for use in fish and

if successful would provide significant advantages over other techniques, enabling the production of non-mosaic transgenics and targeted integration. For example, ES-like cells exhibiting pluripotent characters have been derived from blastula stage zebrafish cells and chimeric fish resulted when these cells were introduced into zebrafish embryos (Sun *et al.*, 1995). Although blastomeres from chinook salmon have been transferred into rainbow trout (Cloud and Kersten, 1994) there is likely to be considerable development time before this technique is applied in salmon.

Another option for transferring genes into fish is the direct injection of DNA into tissue. This has been carried out successfully in muscle cells of mice (Wolf *et al.*, 1990; Wells, 1993) and fish (Rahman and Maclean, 1992; Anderson *et al.*, 1996). The technique is easy to perform and results are obtained quickly, but only certain tissues, such as muscle, may express DNA transferred this way, and the constructs used will be limited to the promoters active in these tissues (Rahman and Maclean, 1992). The possibility of using a similar method to directly introduce genes into germ line cells, thereby eliminating a generation normally required to produce a transgenic line, has been suggested (Gong and Hew, 1995).

In summary, it is clear from the results presented in this thesis that electroporated salmon sperm can successfully take up DNA, and are efficient vectors for transferring genes into embryos. However, the DNA introduced in a naked state does not persist and the levels decrease as the fish develop. Nevertheless, as outlined above, there are many techniques that could be used to enhance the persistence and integration of DNA following sperm transfer. Therefore, if combined with such a method(s), sperm electroporation has the potential to be further developed as an efficient mass gene transfer method in salmon.

Acknowledgements

I would like to thank my supervisor Dr. Frank Sin for his guidance, support and enthusiasm over the years. I am very grateful to Dr. Jane Symonds for her advice and help with preparation of this thesis. I would also like to thank Dr. Liz Allison and Dr. Drusilla Mason. Thanks to everyone in the Molecular Biology/Genetics lab for their friendship support and interesting discussions, including Simon Bulman, Jenny Khoo, Kirstie Murdoch, and Melanie North. Thanks are also due to Jan McKenzie, Terry Williams and Barbara Cottrell for their photographic work. I am grateful to the staff of the Zoology Department especially Lyn de Groot, Franz Ditz, Dave Greenwood, Bruce Lingard, Linda Morris, Tracey Robinson and Roy Thompson.

I thank the Foundation for Research Science and Technology and the Department of Zoology for supporting the salmon genetics research. Thanks also go to the National Institute of Water and Atmospheric Research for the use of the Silverstream hatchery and to Lindsey Hawke for rearing the salmon.

I thank Dr. Robert Devlin and the Department of Fisheries and Oceans Canada for supporting my visit to Vancouver and the people at the West Vancouver Molecular Genetics Laboratory, especially Timothy Yesaki, Carlo Biagi and Duane Smailus.

Thanks to my parents and family for their continued support. Special thanks must go to my wife Jane for her love, friendship and support.

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Appendix 1. Oligonucleotide primer sequences and polymerase chain reaction conditions.

Primer pair	Primer sequences (5' to 3')	Purpose	Magnesium chloride concentration (mM)	Annealing temperature (°C)
P322 LacZ	ACCTCTGACTTGAGCGTCGA ATTCAGGCTGCGCAACTGTT	pRSV-lacZ detection, amplifies 1300 bp product	2	58
Lac2 Lac3	GACCATGATTACGGATTAC GTAGCCAGCTTTCATCAACA	Internal product, used to detect 1300 bp product, amplifies 166 bp product	1.5	58
CAT1 CAT2	AATAAGCGGATGAATGGCAG TCCTCTCAAGTAGAGGAGGT	pSV2CAT detection, amplifies 935 bp product	2	58
MCH1 MCH2	TGATTCAAGTCAGAAGCGGGA GACAAGATGCAGCATAGTAG	Positive control, amplifies 311 bp of the chinook salmon melanin concentrating hormone gene	2	58
ISV1 ISV2	AAATAGGCGTATCACGAGGC AGTGGGTTACATCGAACTGG	Inverse PCR primers to detect integration of pRSV-lacZ DNA	2	58
IPRL1 IPRL2	TAGAACCCTGAGGAAACCTG GAAGTGCTGAGTGAGTGAAG	Inverse PCR positive control, specific to chinook salmon prolactin gene	1.5	58
MT-1 GH19	TCAGGAATTCTGATTAAGTTTTGTATAGT GTAAATTGTATTAATGGT	OnMTGH1 detection, amplifies 400 bp product	1.5	55

Appendix 2.

This appendix consists of the reprints of the following papers:

Symonds, J.E., Walker, S.P. and Sin, F.Y.T. (1994a). Electroporation of salmon sperm with plasmid DNA: Evidence of enhanced sperm/DNA association. *Aquaculture* 119: 313-327.

Symonds, J.E., Walker, S.P., Iris, I.L. and Sin, F.Y.T. (1994b). Development of a mass gene transfer method in chinook salmon: optimisation of gene transfer by electroporated sperm. *Molecular Marine Biology and Biotechnology* 3(2): 104-111.

Walker, S.P., Symonds, J.E., Sin, I.L. and Sin F.Y.T. (1995). Gene transfer by electroporated chinook salmon sperm. *Journal of Marine Biotechnology* 3: 232-234.

The paper published in *Aquaculture* (Symonds *et al.*, 1993a) covers work presented in Chapter 2. I participated in all parts of this study, including the initial electroporation and subsequent sperm washing, DNAase I digestion, and DNA extraction. The Southern and PCR analysis were exclusively carried out by myself. However, the HB101 transformation and dot blot analysis were carried out by Dr. Jane Symonds.

The second paper (Symonds *et al.*, 1993b), describes the analysis of salmon sperm gene transfer carried out in the 1992 breeding season. Dr. Jane Symonds assisted with the PCR analysis of fry from the 1992 season and completed analysis of fry DNA samples from the 6 treatments using linear pRSV-lacZ DNA (see Table 3.1, page 54).

The final paper, published in *Journal of Marine Biotechnology* (Walker *et al.*, 1995), contains a summary of the all the previous work on salmon sperm electroporation and the preliminary results of experiments carried out in the 1993 season. The experiments carried out in 1993 were carried out exclusively by myself.

Signed: 

Date: 24-3-97

Reprinted from

Aquaculture

Aquaculture, 119 (1994) 313–327
Elsevier Science B.V., Amsterdam

AQUA 30092

Electroporation of salmon sperm with plasmid DNA: evidence of enhanced sperm/DNA association

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(Accepted 23 August 1993)



Aquaculture

An international journal devoted to research on the exploration, improvement and management of all aquatic food resources, both floristic and faunistic, from freshwater, brackish and marine environments, related directly or indirectly to human consumption.

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Publication information: *Aquaculture* (ISSN 0044-8486). For 1994 volumes 119-128 are scheduled for publication. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where air delivery via SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues must be made within six months of our publication (mailing) date. Please address all your requests regarding orders and subscription queries to: Elsevier Science B.V., Journal Department, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. Tel.: (+31-20)5803642, fax: (+31-20)5803598.

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AQUA 30092

Electroporation of salmon sperm with plasmid DNA: evidence of enhanced sperm/DNA association

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(Accepted 23 August 1993)

ABSTRACT

We examined the effect of electroporation on salmon sperm/DNA association using bacterial transformation, the polymerase chain reaction, dot blot and Southern analysis. Sperm were electroporated in the presence of circular or linear pRSV-lacZ DNA at 20 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, with field strengths of 625 V/cm or 1000 V/cm, and with either a single or double pulse. Electroporation enhanced sperm retention of circular and linear pRSV-lacZ DNA, and retention was greatest at the higher field strength with two pulses. Sperm/DNA retention showed a direct relationship with DNA concentration and was greater at the higher pRSV-lacZ concentration (100 $\mu\text{g/ml}$). The sperm/DNA association was strong as it resisted repeated washing. However, most of the pRSV-lacZ DNA remained on the outside of the sperm as it was DNase sensitive.

INTRODUCTION

The transfer of foreign DNA into mammals (for example see Palmiter et al., 1983; Pursel et al., 1989; Wall and Seidel, 1992) and fish (for reviews see Maclean et al., 1987; Ozato et al., 1989; Chen and Powers, 1990; Maclean and Penman, 1990; Fletcher and Davies, 1991; Thorgaard, 1992) has been well documented over the last few years. Most studies have employed embryo microinjection for gene transfer. However, given the difficulties encountered with this technique, alternative methods have been tried with varying degrees of success, such as retroviral transfer (Jahner et al., 1985), the use of liposomes (Schaffer-Ridder et al., 1982), microprojectile bombardment (Zelenin et al., 1991), and electroporation of fertilised fish eggs (Inoue et al., 1990).

Brackett et al. (1971) reported the uptake of SV40 DNA by rabbit sperm and its transfer to rabbit ova through fertilisation. Studies on porcine sperm/

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DNA association showed that sperm incubated with foreign DNA were capable of binding this DNA, and that the binding was strong as it resisted multiple washing steps, gradient centrifugations, and prolonged dialysis (Horan et al., 1991). Perez et al. (1991) demonstrated that bovine sperm were capable of transferring foreign DNA into blastocysts. Gagne et al. (1991) studied the effects of electroporation of bovine sperm on DNA uptake and found that electroporation enhanced foreign DNA uptake by sperm cells. In addition this foreign DNA was detected in 5-day-old bovine embryos when they were fertilised by sperm electroporated with the foreign DNA (Gagne et al., 1991).

The use of sperm for gene transfer is particularly appealing in the field of fish transgenics, as microinjection of fish eggs has met with difficulties due to the hard chorion and the opaqueness of the eggs. Recently Khoo et al. (1992) showed that zebrafish sperm, when mixed with foreign DNA, were capable of transferring the DNA into zebrafish embryos. Müller et al. (1992) were able to use electroporated sperm to transfer DNA into common carp (*Cyprinus carpio* L.), African catfish (*Clarias gariepinus*) and tilapia (*Oreochromis niloticus*); no transfer was observed using non-electroporated sperm.

Studies in our laboratory on the transfer of circular pRSV-lacZ DNA into chinook salmon (*Oncorhynchus tshawytscha*) embryos using sperm as a vector have shown that electroporation enhances DNA transfer, and that the frequency of transfer is dependent on field strength and pulse length (Sin et al., 1993). In the present study we have investigated the uptake of circular and linear pRSV-lacZ plasmid by electroporated and non-electroporated chinook salmon sperm, and have examined the effect of electroporation conditions on this uptake. The effect of digesting the sperm with DNase after incubation with pRSV-lacZ DNA was also examined to see if any DNA was entering the sperm. Since ultimately gene transfer methods aim for expression of the transferred DNA, it is important that the introduced DNA remain intact during the uptake process. Thus, three different DNA detection methods were employed as it was important not only to be able to detect low amounts of pRSV-lacZ DNA, but also to determine the condition of the DNA detected.

MATERIALS AND METHODS

Plasmid DNA preparation and analysis

The 6.9 kb plasmid, pRSV-lacZ (Gorman et al., 1983), contains the bacterial gene *lacZ* (beta-galactosidase) under the control of the long terminal repeat (LTR) of the Rous Sarcoma virus (Fig. 1). pRSV-lacZ DNA was prepared using the alkaline lysis procedure (Sambrook et al., 1989), and linearised by cleavage at the single *Sca* I site within the pBR322 sequence.

Plasmid DNA from single HB101 colonies obtained after transformation with sperm DNA was isolated using the alkaline lysis mini-preparation method

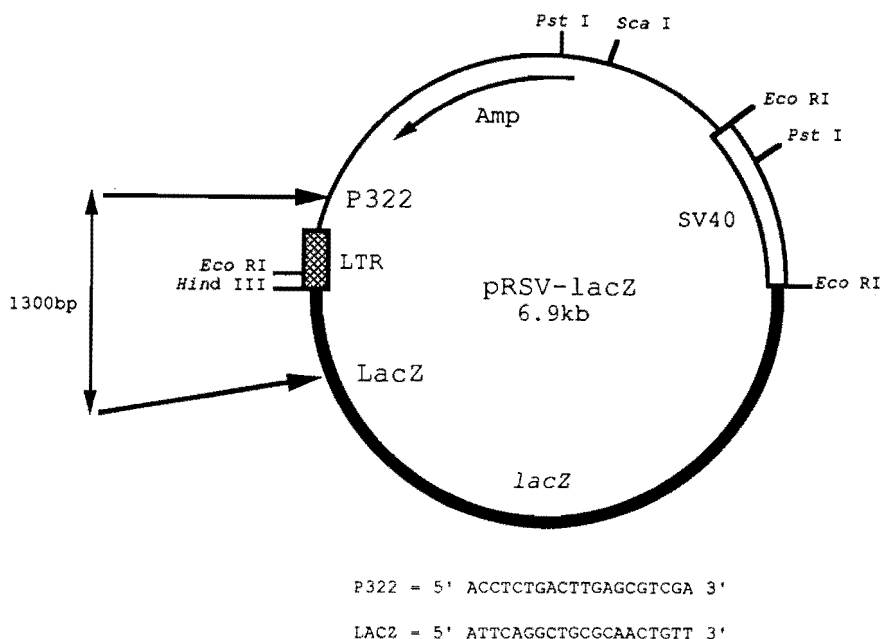


Fig. 1. Map of pRSV-lacZ. *lacZ* gene — solid thick line; Rous Sarcoma Virus LTR — shaded box; SV40 processing signal — open box. The positions of the two 20-mer oligonucleotide primers (P322 and LacZ) used in the PCR analysis are given, along with their sequence and the size of the amplified product (1300 bp). The restriction sites for *Eco* RI, *Hind* III, *Pst* I, and *Sca* I are indicated. Not drawn to scale.

(Sambrook et al., 1989), and digested with the restriction endonucleases *Eco* RI and *Pst* I (Boehringer Mannheim) for electrophoretic analysis on 1% agarose gels.

Electroporation and sperm preparation

Fresh sperm were obtained from a single salmon and stored at 0°C until use the same day. 0.5 ml of sperm were mixed with 0.5 ml of HEPES-buffered saline (21 mM HEPES (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose) containing different forms of pRSV-lacZ DNA (see Table 1) and electroporated using different combinations of field strengths, and number of pulses (see Table 1 and Fig. 2). A pulse length of 27.4 ms was used for all the electroporations. A Cell-Porator (Bethesda Research Laboratories) was used for the electroporation with a distance of 4 mm between the electrodes. At least two replicate sperm samples were electroporated for each set of conditions. Following electroporation, the ability of the sperm to be activated was assessed under a light microscope at 400× magnification. Five µl of sperm were placed onto a microscope slide to which a drop of water was

added. If the sperm remained intact then the addition of water immediately initiated sperm motility (Billard and Cosson, 1992).

Non-electroporated sperm were similarly treated with pRSV-lacZ DNA to assess DNA uptake. After electroporation all samples were kept on ice for 10 min to allow DNA uptake and were then spun at 2000 g for 10 min at 4°C. The supernatant (SN) was removed and kept for analysis. The details of the subsequent sperm sample preparation for DNA uptake assessment are outlined in Fig. 2.

The efficiency of the DNase I digestion was tested by incubating 100 µg, 50 µg, 25 µg, and 10 µg of circular pRSV-lacZ in 500 µl DNase digestion buffer with 50 units of DNase I at 37°C for 2.5 h. The reaction was terminated by the addition of 25 µl 0.5 M EDTA and the samples heated at 65°C for 15 min.

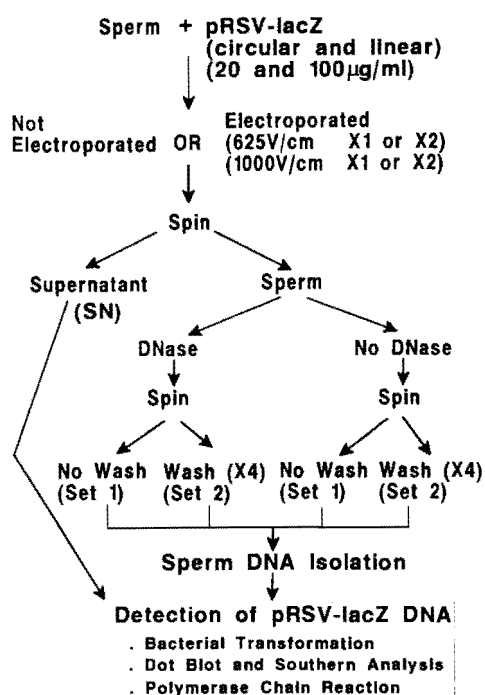


Fig. 2. Outline of the method used to assess sperm DNA uptake. Two types of pRSV-lacZ DNA were used (circular or linear) at a concentration of either 20 µg/ml or 100 µg/ml. The electroporation conditions tested were field strength (625 V/cm or 1000 V/cm) and the number of pulses (1 or 2). After treatment all sperm were pelleted and the supernatant (SN) retained for analysis. All sperm were resuspended in 1 ml of DNase digestion buffer (0.1 M sodium acetate pH 5.0, 5 mM MgSO₄) and half of each sample (500 µl) was digested with 50 units of DNase I for 2.5 h at 37°C. The digested and undigested samples were pelleted and resuspended in HEPES buffer. All sperm samples were once more divided into two aliquots; set 1 was stored at -20°C; set 2 was washed four more times in HEPES buffer (washed samples) and stored at -20°C. DNA was extracted and analysed for the presence of pRSV-lacZ DNA.

Samples without DNase, but containing equivalent DNA amounts, were also treated similarly. Plasmid DNA was recovered from the samples by ethanol precipitation and its presence assessed by agarose gel electrophoresis and HB101 transformation.

DNA extraction

DNA was extracted from 50 μ l of all sperm samples using a modification of the method of Penman et al. (1990), to give a final volume of 150 μ l in TE8 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Detection of pRSV-lacZ DNA in the sperm samples

Transformation of E. coli strain HB101. To assess the association of circular pRSV-lacZ DNA with electroporated and non-electroporated sperm, transformation of the *E. coli* strain HB101 was chosen. 100 μ l of the supernatant samples and 100 μ l of each sperm DNA sample, extracted from all samples incubated with circular pRSV-lacZ DNA, were used in the transformation assay (Sambrook et al., 1989). The number of transformants obtained for each set of treatments was compared using the nonparametric Mann-Whitney test (Zar, 1984).

The polymerase chain reaction (PCR). PCR was used to detect pRSV-lacZ in the DNA samples from the washed sperm (set 2, see Fig. 2) incubated with circular and linear pRSV-lacZ. Two 20-mer oligonucleotide primers within pRSV-lacZ were used to amplify a 1300 bp fragment (Fig. 1). As a control, and to assess the ability of the extracted DNA to be amplified, a portion (311 bp) of the melanin concentrating hormone gene (MCH) of chinook salmon (Minth et al., 1989) was amplified in a separate PCR reaction. Oligonucleotide primers were obtained from the Christchurch Medical School.

DNA amplification was carried out by the method of Saiki et al. (1988) with minor modifications. Each amplification mixture (25 μ l) contained 2.0 μ g of sperm DNA. PCR amplification was performed with a Grant Autogene II thermocycling waterbath and consisted of 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C. A final extension of 7 min was included. The PCR products were analysed by 2% agarose gel electrophoresis.

Dot blot and Southern analysis. For dot blot analysis 1 μ g (– DNase, 20 μ g/ml samples), 0.5 μ g (– DNase, 100 μ g/ml samples) or 10 μ g (+ DNase samples) of sperm DNA in 10 μ l of TE8 buffer was applied directly onto dry nitrocellulose filters (Schleicher and Schuell) (Aquadro et al., 1986). DNA concentration was standardised by comparison of the fluorescence intensity of the DNA on agarose gels with DNA standards (Sambrook et al., 1989).

For Southern blots, 4 μ g (– DNase samples) or 20 μ g (+ DNase samples)

of undigested sperm DNA, and 4–10 μg of the DNA from the electroporated sperm samples (– DNase only) digested with the restriction enzymes *Hind* III and *Eco* RI (Boehringer Mannheim), were electrophoresed in a 1% agarose gel and transferred to Zeta-probe nylon membrane (Biorad).

Hybridisation was carried out at 65°C in 1 mM EDTA, 0.5 M NaH_2PO_4 , pH 7.2, 7% SDS and 0.1% (w/v) disodium pyrophosphate, using [α - ^{32}P] dCTP nick translated pRSV-lacZ DNA as a probe (Rigby et al., 1977). Filters were washed at high stringency and exposed to X-ray film (Amersham Hyperfilm) with intensifying screens from 12 h to 2 weeks at –80°C.

RESULTS

Effect of electroporation on sperm

Electroporation had no observable effect on the ability of the sperm to be activated, as sperm motility was initiated in sperm samples from all treatments immediately after electroporation, although the percentage of motile sperm was not quantified. No evidence of sperm aggregation or lysis was observed at any stage of the sample preparation, and it was assumed that sperm clumping was not a problem during the washing procedure.

Bacterial transformation

Bacterial transformation was used to assess the association of circular pRSV-lacZ DNA with electroporated and non-electroporated sperm. The HB101 transformation results are summarised in Table 1. Prior to sperm DNA extraction the sperm were pelleted and the supernatant (SN) removed for transformation analysis (Fig. 2). Only supernatant from the non-electroporated sperm samples produced a significant number of transformants, the number increasing when more pRSV-lacZ DNA (100 μg) was used to electroporate the sperm. Only two out of the 14 supernatants analysed from the electroporated sperm samples produced a single transformant (Table 1).

The presence of pRSV-lacZ DNA in the sperm DNA samples was also indicated by the growth of HB101 transformants (Table 1). When non-electroporated sperm DNA samples were used, irrespective of the pRSV-lacZ DNA concentration used, only a small number of transformants was observed. In contrast all the electroporated (20 $\mu\text{g}/\text{ml}$ pRSV-lacZ) sperm DNA samples produced at least 10 HB101 colonies per replicate, and at the higher pRSV-lacZ concentration (100 $\mu\text{g}/\text{ml}$) the number of transformants increased (Table 1).

DNase digestion of the sperm prior to DNA extraction had a significant effect on the number of transformants produced, and for most of the sperm samples electroporated with 20 μg of circular pRSV-lacZ no transformants were obtained following digestion (Table 1). Three out of four of the DNA

TABLE 1

Total number of HB101 colonies detected after transformation with supernatant (SN) and sperm DNA^a

Electroporation conditions – field strength (V/cm)	Number of pulses	Amount of DNA (μ g)	DNase + or –	SN (N)	Sperm DNA Set 1 (N)	Sperm DNA Set 2 (N=4)
No shock	0	20	–	45 (5)	0 (3)	0
No shock	0	20	+		0 (3)	0
625	1	20	–	1 (3)	36 (3)	22
625	1	20	+		1 (3)	0
625	2	20	–	0 (3)	51 (3)	30
625	2	20	+		0 (3)	1
1000	1	20	–	0 (3)	32 (3)	37
1000	1	20	+		0 (3)	0
1000	2	20	–	0 (3)	43 (3)	42
1000	2	20	+		0 (3)	0
No shock	0	100	–	80 (2)	6 (4)	NT
No shock	0	100	+		0 (4)	NT
1000	2	100	–	1 (2)	120 (4)	286
1000	2	100	+		3 (4)	1

^aSet 1 = non-washed sperm samples. Set 2 = washed sperm samples. DNase (+ or –) indicates whether or not the sperm sample was treated with DNase prior to DNA extraction. The total number of transformants observed for all the replicates of a particular treatment is given. The number of replicates (N) is in parentheses and refers to the number of independent sperm DNA samples analysed. NT = not tested.

samples extracted from sperm electroporated with 100 μ g/ml pRSV-lacZ produced a single transformant after DNase digestion.

For the non-DNase digested sperm DNA samples, the number of transformants obtained was independent of the field strength used. Doubling the number of pulses applied slightly increased the number of transformants obtained, but this difference was not significant ($P > 0.2$). Additional washing of the sperm in HEPES buffer did not reduce the number of transformants significantly ($P > 0.2$). Given that transformants were detected in the washed sperm samples (set 2), only these samples were analysed using the other detection methods.

pRSV-lacZ DNA was recovered from some of the HB101 transformants produced using DNA from the electroporated sperm samples (set 2) and was digested with *Pst* I and *Eco* RI. Identical fragments to control pRSV-lacZ DNA (Fig. 1) were obtained for these plasmid DNA samples. The DNA recovered from these colonies was also amplified using the two PCR primers within pRSV-lacZ (Fig. 1). All samples produced a single amplification product of 1300 bp.

In order to exclude the possibility of transformants being produced as a

result of incomplete DNase digestion, control DNase digestions were carried out. No pRSV-lacZ DNA could be detected after DNase digestion and no HB101 transformants were obtained when the digested samples were used for transformation.

The polymerase chain reaction (PCR)

PCR was used to screen for the presence of pRSV-lacZ DNA in the DNA extracted from all washed sperm samples (circular and linear pRSV-lacZ, Fig. 2) using the two primers shown in Fig. 1. The PCR results are shown in Fig. 3. The 1300 bp pRSV-lacZ PCR product was detected in all non-electroporated and electroporated, non-DNase digested, sperm DNA samples and also in most of the DNase digested samples (Fig. 3). Two of the DNase digested treatments failed to produce a detectable 1300 bp PCR product: the 1000 V/cm 2 pulse samples electroporated with 20 µg/ml circular (lane 12) and 20 µg/ml linear pRSV-lacZ DNA (lane 24). In all other DNA samples extracted from DNase digested sperm the 1300 bp PCR product was observed, although the intensity of the amplified product was reduced in these samples compared to the non-DNase digested samples (Fig. 3). Fig. 3 shows only the results from one sperm DNA sample of each treatment, all other replicates (2 or 3) produced the same results.

The control salmon MCH PCR product (311 bp) was observed in all the

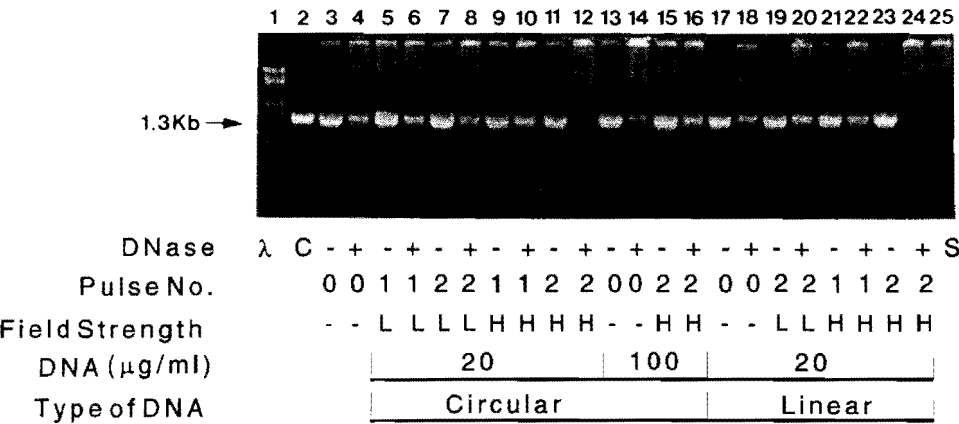


Fig. 3. PCR products detected in the DNA samples from sperm incubated with circular and linear pRSV-lacZ DNA using the primers P322 and LacZ. Lane 1, lambda DNA digested with *Eco* RI and *Hind* III (fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 2, pRSV-lacZ DNA 1300 bp PCR product (positive control); 25, salmon sperm DNA only (negative control). The electroporation conditions (L=625 V/cm, H=1000 V/cm), the amount (20 µg or 100 µg/ml), and type of pRSV-lacZ DNA used (circular or linear), DNase digestion (– or +), and number of pulses (0, 1 or 2) are indicated for each lane.

sperm samples analysed (data not shown), showing that the sperm DNA could be successfully amplified and that the PCR reactions were reliable.

Dot blot and Southern analysis

Dot blot analysis was used to detect pRSV-lacZ DNA in all the washed sperm DNA samples (data not shown). Almost no pRSV-lacZ DNA (less than 50 pg) was detected in the DNA samples from the non-electroporated sperm. In the samples electroporated with circular pRSV-lacZ DNA (20 µg/ml) the amount of pRSV-lacZ DNA detected increased with increasing field strength and number of pulses, and more pRSV-lacZ DNA was detected when 100 µg of pRSV-lacZ DNA was used for the electroporation. DNase digestion of the electroporated sperm reduced the amount of pRSV-lacZ DNA detected

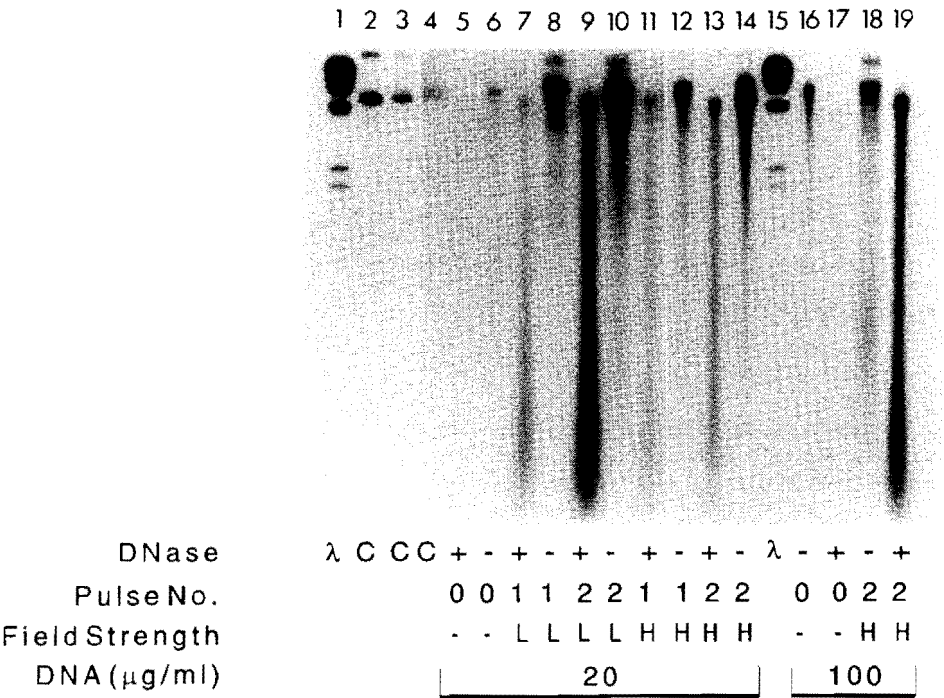


Fig. 4. Southern analysis of DNA extracted from sperm incubated with circular pRSV-lacZ DNA and probed with pRSV-lacZ nick-translated DNA. Lanes 1 and 15, lambda DNA digested with *Hind* III (fragment sizes (bp): 23130, 9416, 6557, 4361, 2322, 2027, 564, 125); 2, pRSV-lacZ DNA (1 ng); 3, pRSV-lacZ DNA (500 pg); 4, pRSV-lacZ DNA (100 pg). The electroporation conditions (*L*=625 V/cm, *H*=1000 V/cm), the amount of pRSV-lacZ DNA used (20 µg or 100 µg/ml), DNase digestion (– or +), and number of pulses (0, 1 or 2) are indicated for each lane. All samples are from the same membrane, but lanes 1–3 and 12–19 were exposed to X-ray film for 4 days, and lanes 4–11 for 14 days.

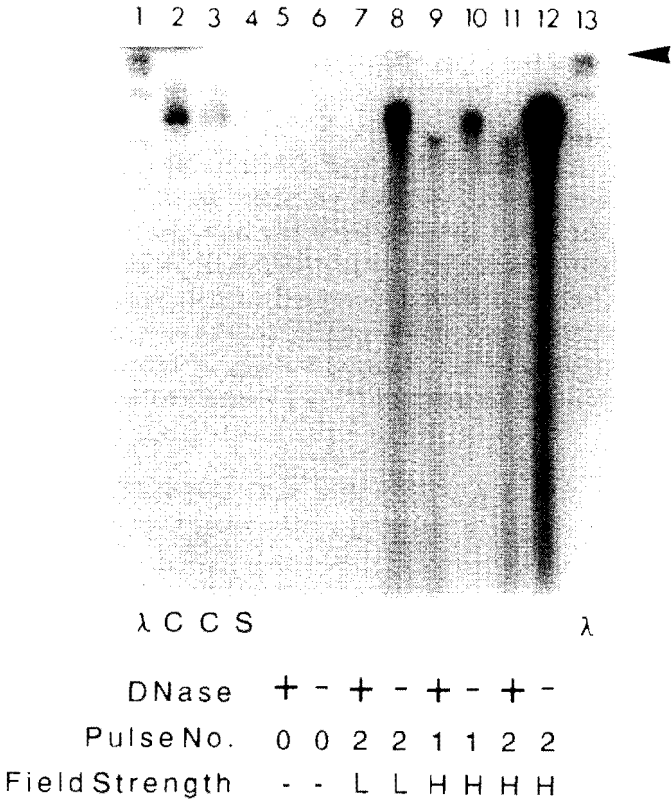


Fig. 5. Southern analysis of DNA extracted from sperm incubated with 20 µg/ml linear pRSV-lacZ DNA and probed with pRSV-lacZ nick-translated DNA. Lanes 1 and 13, lambda DNA digested with *Hind* III (fragment sizes (bp): 23130, 9416, 6557, 4361, 2322, 2027, 564, 125); 2, pRSV-lacZ DNA (1 ng); 3, pRSV-lacZ DNA (400 pg); 4, salmon sperm DNA only (20 µg) (negative control). The electroporation conditions (L=625 V/cm, H=1000 V/cm), DNase digestion (- or +), and number of pulses (0, 1 or 2) are indicated for each lane. The membrane was exposed to X-ray film for 14 days. The arrow shows the position of the high molecular weight band present in lanes 8 and 12.

approximately ten-fold, and the amount of pRSV-lacZ DNA remaining after DNase digestion of the sperm appeared to increase with increasing field strength and number of pulses. Dot blot analysis of the DNA extracted from sperm electroporated with linear pRSV-lacZ (20 µg/ml only) showed similar results.

All the sperm DNA samples used for the dot blots were subsequently used for Southern analysis. In Fig. 4 (DNA from sperm incubated with circular pRSV-lacZ DNA) all non-electroporated samples produced only a faint signal following hybridisation to pRSV-lacZ DNA (lanes 5, 6, 16 and 17). DNA

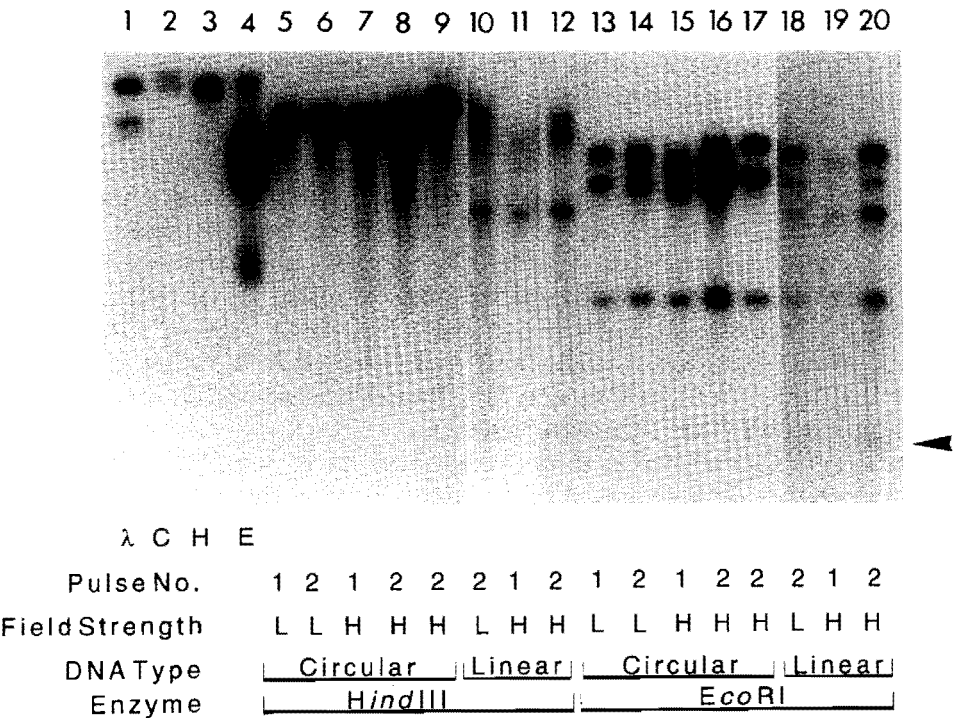


Fig. 6. Southern analysis of sperm DNA digested with *Eco* RI and *Hind* III. Lane 1, lambda DNA digested with *Eco* RI and *Hind* III (fragment sizes (bp): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125); 2, undigested pRSV-lacZ DNA (1 ng); 3, *Hind* III digested pRSV-lacZ DNA (1 ng); 4, *Eco* RI digested pRSV-lacZ DNA (5 ng). Lanes 5 to 12 contain sperm DNA digested with *Hind* III. Lanes 13 to 20 contain sperm DNA digested with *Eco* RI. The electroporation conditions (L=625 V/cm, H=1000 V/cm), type of pRSV-lacZ DNA used (circular or linear), and number of pulses (0, 1 or 2) are indicated for each lane. Lanes 9 and 17 contain DNA extracted from sperm electroporated with 100 µg/ml pRSV-lacZ DNA, all other lanes contain DNA from sperm electroporated with 20 µg/ml. The arrow shows the position of the 0.5 kb band observed in lanes 18, 19, and 20. All samples are from the same membrane, but lanes 1-9 and 12-17 were exposed to X-ray film for 12 h, and lanes 10-11 and 18-20 for 24 h.

migrating similarly to control pRSV-lacZ DNA was detected in all the non-DNase digested electroporated samples (lanes 8, 10, 12, 14 and 18) and overall there appeared to be more pRSV-lacZ DNA present at the higher field strength (lane 14) (lanes 4 to 11 were exposed to X-ray film 3.5 times longer than lanes 12 to 19).

All DNA samples extracted from DNase treated electroporated sperm (circular pRSV-lacZ DNA samples) produced a band of around 4-5 kb as well as a smear below this (Fig. 4, lanes 7, 9, 11, 13 and 19). The intensity of this

band increased with increasing field strength and pulse number, and was the darkest in the sample electroporated with 100 μ g of pRSV-lacZ DNA (lane 19) even when the exposure time was shorter.

The DNA samples from sperm electroporated with linear pRSV-lacZ DNA (Fig. 5) showed similar results. For the non-DNase treated sperm samples to which two pulses had been applied, two bands were observed (lanes 8 and 12) and more DNA was detected at the higher field strength (lane 12). For the single pulse sample (lane 10, 1000 V/cm) only one band was observed, and the hybridisation signal was much fainter. The dark bands visible in lanes 8, 10 and 12 had similar mobilities to pRSV-lacZ DNA (lanes 2 and 3). Following DNase digestion, a single band was observed in the samples electroporated at 1000 V/cm (lanes 9 and 11). No DNA was detected in the 625 V/cm sample after DNase digestion (lane 7).

The non-DNase treated electroporated sperm DNA samples were digested with the restriction enzymes, *Hind* III and *Eco* RI (Fig. 6). After *Hind* III digestion a single band (6.9 kb) was expected for the samples electroporated with circular pRSV-lacZ if no changes to the pRSV-lacZ plasmid had occurred (see Fig. 1). In all samples a single band was observed, although the relative migration of this band varied between the different electroporation conditions (lanes 5 to 9). Three fragments were expected after *Eco* RI digestion (3.3 kb, 2.4 kb, and 1.2 kb), and despite a slight difference in migration between the samples (lanes 13 to 17), the overall fragment pattern was similar to that obtained for the control pRSV-lacZ plasmid (lane 4).

After *Hind* III digestion of DNA from samples electroporated with linear pRSV-lacZ, two fragments were expected (4.9 kb and 2.0 kb) (Fig. 6, lanes 10 to 12). The sample treated with a single pulse of 1000 V/cm produced two bands (lane 11), whereas the two sperm samples treated with double pulses (lanes 10 and 12) produced an additional band. Four fragments were expected following *Eco* RI digestion (3.3 kb, 1.9 kb, 1.2 kb, and 0.5 kb). In the single pulse sample (lane 19) four bands were observed, whereas in the double pulse samples (lanes 18 and 20) five bands were detected.

DISCUSSION

Overall the results indicate that electroporation enhances salmon sperm/DNA association. The HB101 transformation, dot blot, and Southern analyses detected virtually no pRSV-lacZ DNA (circular or linear) in the DNA samples extracted from the non-electroporated sperm. The presence of pRSV-lacZ DNA in the supernatant from these samples implied that the DNA was easily removed from the non-electroporated sperm. In contrast the PCR analysis revealed no difference between the non-electroporated and electroporated samples, and probably reflects the greater sensitivity of this technique and its ability to detect femtogram amounts of DNA. pRSV-lacZ DNA was

detected by all four methods in the electroporated sperm DNA samples, and the binding of this DNA to the sperm appeared to be strong as it resisted multiple washing steps.

DNase was added to the sperm samples to degrade any pRSV-lacZ DNA remaining in solution or only loosely attached to the surface of the sperm. Although the bacterial transformation indicated that most of the pRSV-lacZ DNA was removed after DNase digestion, the PCR, dot blots and Southern blots showed that some pRSV-lacZ DNA remained in the sperm DNA samples. Therefore since not all pRSV-lacZ DNA was removed by exhaustive DNase digestion, some of the pRSV-lacZ DNA may be entering the sperm following electroporation. Alternatively, association of the pRSV-lacZ DNA with the sperm plasma membrane may be sufficient to protect the DNA from contact with DNase. Gagne et al. (1991) conducted a similar DNA uptake study with electroporated bovine sperm, and showed that the DNA remaining after DNase digestion was, at the least, partly embedded in the sperm plasma membrane, if not completely inside it.

The dot blot and Southern analysis showed an increase in the amount of pRSV-lacZ DNA associated with the non-DNase and DNase treated sperm when a double pulse of 1000 V/cm was applied. In contrast no pRSV-lacZ DNA was detected by PCR in these DNase digested samples when electroporated with 20 $\mu\text{g}/\text{ml}$ pRSV-lacZ DNA, although a product was obtained when 100 $\mu\text{g}/\text{ml}$ pRSV-lacZ DNA was used for the electroporation. This suggests that under these conditions a proportion of the pRSV-lacZ DNA present was altered, and at the low concentration the amount of intact DNA remaining was below the sensitivity of PCR. The exact nature of the change to the pRSV-lacZ DNA is not known, and could be due to the loss of the primer binding sites or other alterations to the DNA under these conditions. Similarly, the number of HB101 transformants obtained did not increase when the field strength and pulse number was increased, although more pRSV-lacZ DNA was present. Bacterial transformation is the least sensitive of the methods we employed and selects for a functional ampicillin gene. Therefore any pRSV-lacZ DNA carrying a rearrangement in this region would not be detected. By contrast the condition of the DNA is not important when DNA hybridisation techniques are used, and this could account for the different results obtained with the various methods we employed.

In transgenic studies the form of the DNA being introduced into the host is important. In this study we were unable to detect any difference between the samples electroporated with circular or linear pRSV-lacZ DNA, and for both DNA types the amount of pRSV-lacZ DNA associated with the sperm increased with increasing field strength and number of pulses. Following restriction enzyme digestion of the pRSV-lacZ DNA recovered from sperm electroporated with linear pRSV-lacZ DNA, extra fragments were observed in the sperm samples electroporated with a double pulse. However, these ex-

tra fragments can be explained if the linear plasmid had re-ligated at the *Sca* I site; no other rearrangements were observed.

Other studies in cattle (Gagne et al., 1991) and fish (Müller et al., 1992; Sin et al., 1993) have shown that sperm electroporation increases gene transfer efficiencies. Sin et al. (1993) found that the transfer of pRSV-lacZ into chinook salmon fry was dependent on field strength and pulse length, and below 625 V/cm no gene transfer was observed. This is consistent with our findings that increasing the field strength enhances sperm/DNA association. Overall it appears that while incubation of sperm with DNA is sufficient to allow sperm DNA uptake (Perez et al., 1991; Khoo et al., 1992), electroporation can greatly enhance sperm/DNA association and lead to increased gene transfer frequencies.

ACKNOWLEDGEMENTS

This study was supported by grants from the Department of Zoology, the University of Canterbury Staffing Committee, New Zealand Lottery Grants Board and the University Grants Committee of New Zealand to F.Y.T.S.. J.E.S. is supported by a UGC postdoctoral fellowship. The plasmid, pRSV-lacZ, was a generous gift from Dr L.A. Allison.

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Aquaculture, Vol. 119, No. 1, pp. 101–106 (1994)

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0044-8486/94/\$07.00

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PRINTED IN THE NETHERLANDS

Development of a mass gene transfer method in chinook salmon: optimization of gene transfer by electroporated sperm

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Abstract

We examined the effect of electroporation on the viability of chinook salmon (*Oncorhynchus tshawytscha*) sperm, as well as the ability of the electroporated sperm to transfer plasmid DNA into salmon embryos. Sperm were electroporated in the presence of circular or linear pRSV-lacZ and circular pSV2CAT DNA at 20 $\mu\text{g/mL}$. Field strengths of 625 or 1,000 V/cm and pulse lengths of 18.6 or 27.4 msec were used, with a single, double, or triple pulse. Sperm motility was shown to decrease with increasing field strength, pulse length, and pulse number, and the number of embryos surviving to the fry stage was dependent on the percentage of motile sperm used for fertilization. DNA from 12-week-old fry was analyzed by the polymerase chain reaction (PCR) and Southern analysis for the presence of pRSV-lacZ and pSV2CAT DNA. Electroporation was found to enhance sperm gene transfer, but transfer frequencies varied depending on the electroporation conditions used. Transfer of pRSV-lacZ and pSV2CAT DNA was greatest at the higher field strength (1,000 V/cm) with 2 pulses; transfer efficiencies as high as 85% were achieved. Although pRSV-lacZ and pSV2CAT DNA was detected in the 12-week-old fry using PCR, Southern analysis failed to detect any plasmid DNA in these fry. Although electroporated sperm are capable of transferring the plasmid DNA, the levels of this DNA in the fry are low, and the resulting fry are

mosaic. The lacZ gene was tested for expression, but no expression was evident.

Introduction

Microinjection has been used extensively for introduction of foreign DNA into fish embryos. However, due to the difficulties encountered with this technique in fish, alternative methods have been attempted. These methods include microprojectile bombardment of loach, rainbow trout, and zebrafish embryos (Zelenin et al., 1991); electroporation of the fertilized eggs of medaka, zebrafish, African catfish, rosy barb, red crucian carp, channel catfish, and common carp (Inoue et al., 1990; Buono and Linser, 1992; Inoue, 1992; Powers et al., 1992; Müller et al., 1992; Xie et al., 1993); and zebrafish embryo baekonization (Zhao and Wong, 1991; Zhao et al., 1993). The variable gene transfer efficiencies achieved by these methods ranged from 4% (Inoue et al., 1990) to 70% (Zhao et al., 1993).

Successful uptake and transfer of exogenous DNA by zebrafish sperm cells has been demonstrated (Khoo et al., 1992). Recently, electroporation of fish sperm has been shown to increase gene transfer efficiencies in common carp, African catfish, tilapia (Müller et al., 1993), and chinook salmon (Sin et al., 1993); efficiencies ranged from 2.6 to 10%. Effective gene transfer by electroporation is dependent on a number of electrical and biological parameters (Andreason and Evans, 1988). Studies in our laboratory have shown that the efficiency of DNA uptake by electroporated chinook salmon sperm is dependent on field strength, number of pulses applied, and DNA concentration (Symonds et al., 1994). We have also shown that the frequency of DNA transfer into embryos by electroporated sperm is dependent on field strength and pulse length (Sin et al., 1993).

With the aim of optimizing chinook salmon sperm electroporation, we investigated the effect of different combinations of field strength, pulse length, and pulse number on gene transfer efficiencies. In addition, to assess whether electroporated

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sperm remained capable of fertilizing eggs, sperm motility was estimated for each treatment, and the viability of the embryos fertilized by the electroporated sperm was determined.

Results

Motility of chinook salmon sperm after electroporation

Sperm motility was found to decrease with increasing field strength and pulse length; less than 5% of sperm remained motile when 2 pulses (27.4 msec at 1,000 V/cm) were used (Figure 1). The effect of applying up to 5 pulses of 18.6 or 27.4 msec at 1,000 V/cm to the sperm was also tested. Motility was found to decrease with increasing pulse number (data not shown); 5 pulses of 18.6 msec and 4 pulses of 27.4 msec reduced sperm motility to 0%. The number of eggs surviving to the fry stage was shown to be dependent on the percentage of motile sperm present in each sample used to fertilize 300 eggs ($r = 0.77$; $p < 0.002$) (Figure 2).

Detection of pRSV-lacZ and pSV2CAT DNA in fry using PCR

The presence of pRSV-lacZ and pSV2CAT DNA in the 12-week-old fry was determined by the polymerase chain reaction (PCR) using the primers

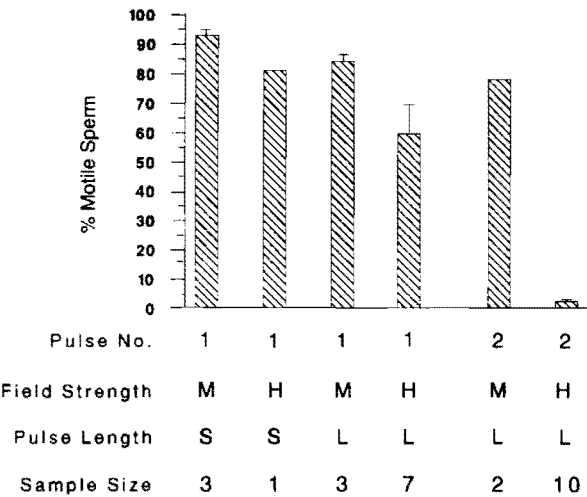


Figure 1. Effect of electroporation on sperm motility. Sperm were electroporated using a high (1,000 V/cm) or medium (625 V/cm) field strength, with a single (1) or a double pulse (2) of short (18.6 msec) or long (27.4 msec) duration. The number of individual sperm samples analyzed is indicated (sample size).

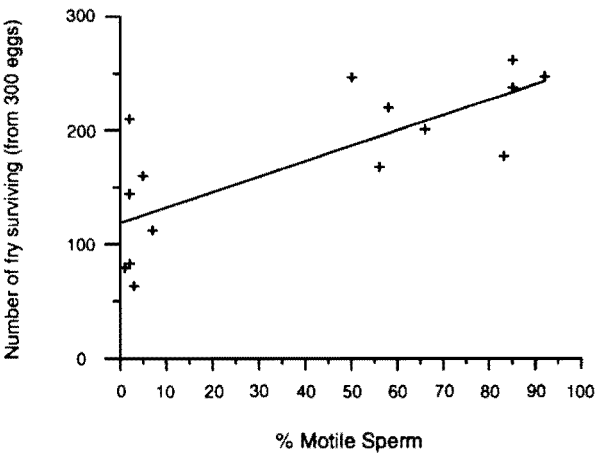


Figure 2. The number of fry surviving from 300 eggs as a function of the percentage of motile sperm present in the sample used for fertilization. Only samples in which more than 1% of the sperm were motile were used for the analysis.

shown in Figure 3. Figure 4 shows representative results of this assay, in which a 1.3-kb DNA fragment was amplified in the fish containing pRSV-lacZ DNA. The results are summarized in Table 1. No transfer of circular or linear pRSV-lacZ DNA was observed when nonelectroporated sperm were used to fertilize salmon embryos. However, transfer of pRSV-lacZ DNA was observed when the sperm were electroporated, and the frequency of transfer was dependent on the electroporation conditions used. DNA topology did not have a noticeable effect on transfer because similar efficiencies of transfer were obtained with circular and linear pRSV-lacZ DNA. When a single pulse was applied to the sperm, the highest transfer efficiencies (39 and 32%) were obtained at the higher field strength (1,000 V/cm) and the lower pulse length (18.6 msec). Increasing the duration of the pulse to 27.4 msec decreased transfer; however, a higher transfer frequency (44%) was obtained with linear pRSV-lacZ DNA when the sperm were electroporated with 2 pulses of 27.4 msec at 1,000 V/cm. No positive fry were detected when the sperm were electroporated at 625 V/cm with 2 27.4-msec pulses, followed by 1 18.6-msec pulse.

The ionic strength of the buffer in which electroporation is performed is critical (Andreason and Evans, 1988). In this study, we used a final concentration of 0.5X HBS for most of the electroporations. However, to test the effect of the ionic strength of the buffer on transfer efficiencies, we

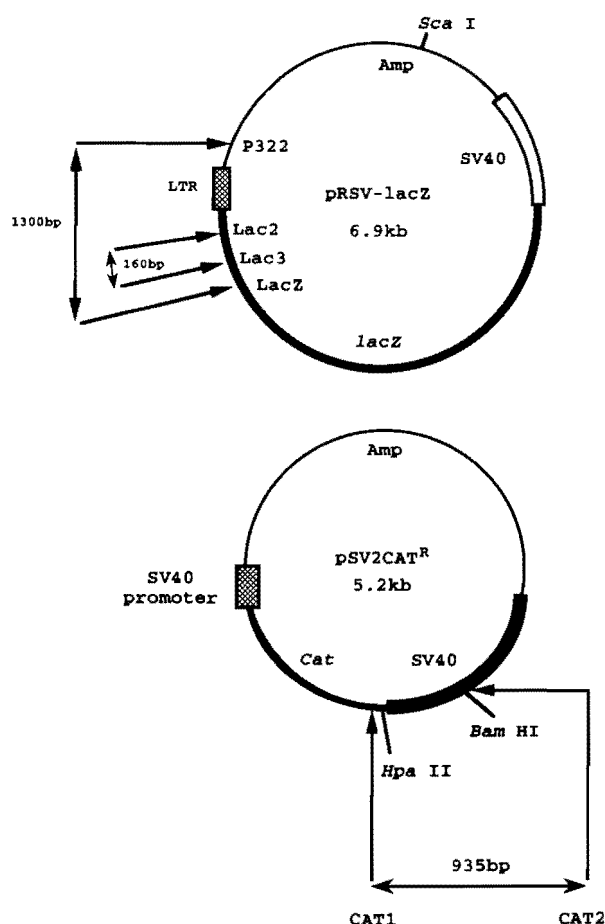


Figure 3. Schematic maps of the plasmids pRSV-lacZ and pSV2CAT. pRSV-lacZ: The *lacZ* gene is shown as a solid thick line, the Rous Sarcoma Virus LTR as a shaded box, and the SV40 processing signal as an open box. The primers P322 and LacZ were used in the PCR analysis to amplify a 1,300-bp fragment in fry containing pRSV-lacZ DNA. The primers Lac2 and Lac3 were used to amplify an internal fragment (160 bp) of the *lacZ* gene for use as a probe. The restriction site for *Sca*I is indicated. pSV2CAT: The *Cat* gene is shown as a thick line, the SV40 early region promoter as a shaded box, and the SV40 processing region as a black box. The primers CAT1 and CAT2 were used in the PCR analysis to amplify a 935-bp fragment. The primer binding sites were removed by digestion with *Hpa*II and *Bam*HI. The maps are not drawn to scale.

carried out 2 of the sperm electroporations (625 V/cm \times 18.6 msec, 625 V/cm \times 27.4 msec, single pulses only) with circular pRSV-lacZ DNA in a final concentration of 1X HBS. Transfer frequencies similar to those observed for 0.5X HBS were observed (see Table 1), although the higher frequency

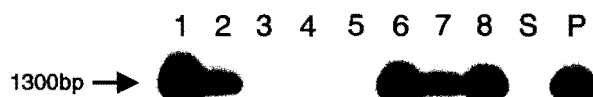


Figure 4. Analysis of fry genomic DNA by PCR and Southern hybridization for the presence of pRSV-lacZ DNA. Lanes 1 to 8: DNA samples from fry (electroporated samples); lane S: salmon sperm DNA (negative control); lane P: pRSV-lacZ (positive control, 1,300 bp). All samples are from the same membrane, but lane P (positive control) was exposed to radiographic film for 2 hours; lanes 1 to 9 were exposed for 5 days.

was observed with the longer pulse length in 1X HBS and with the shorter pulse length in 0.5X HBS.

Following eletroporation with pRSV-lacZ DNA, the sperm samples were routinely incubated on ice for 10 minutes before being used to fertilize the salmon eggs, because this approach is thought to prolong the period when the membrane is permeable to DNA (Spencer, 1991). Following application of 2 pulses of 27.4 msec at 1,000 V/cm, the incubation time on ice was extended to 30 minutes for 1 sperm sample (linear pRSV-lacZ). None of the 21 fry analyzed were found to contain pRSV-lacZ DNA.

The presence of carrier DNA in the cell/DNA solution during electroporation can increase gene transfer efficiencies (Chu et al., 1987; Spencer, 1991). Therefore, we added calf thymus DNA (final concentration 10 μ g/mL) to 1 sperm/pRSV-lacZ DNA (linear) sample before applying 2 pulses of 27.4 msec at 1,000 V/cm to the sperm. None of the 20 fry tested were found to contain pRSV-lacZ DNA.

Unlike pRSV-lacZ DNA, circular pSV2CAT DNA was detected in 15% of the fry that developed from embryos fertilized by nonelectroporated sperm (see Table 1). However, the frequency of transfer was increased when electroporated sperm were used. The highest transfer frequency was 85%, when 2 pulses of 27.4 msec at 1,000 V/cm were applied to the sperm.

As a negative control for the PCR reactions, DNA was extracted from 20 fry that had been produced by simply mixing sperm and eggs, neither of which had come into contact with plasmid DNA. This DNA was then amplified using both sets of plasmid primers (see Figure 3). No plasmid PCR products were detected in these negative control samples.

The control salmon melanin concentrating hormone (MCH) PCR product (311 bp) was observed in

Table 1. Effect of electroporation of sperm on plasmid DNA transfer.

Electroporation conditions: field strength (V/cm) × pulse length (msec)	Number of pulses	HBS: 1 × DNA: LacZ, C. Positive Fry/N (%)	HBS: 0.5 × DNA: LacZ, C. Positive Fry/N (%)	HBS: 0.5 × DNA: LacZ, L Positive Fry/N (%)	HBS: 0.5 × DNA: CAT, C. Positive Fry/N (%)
No shock	0	—	0/18 (0%)	0/20 (0%)	3/20 (15%)
625 × 18.6	1	4/28 (14%)	3/18 (17%)	5/20 (25%)	—
625 × 27.4	1	6/22 (27%)	2/20 (10%)	0/20 (0%)	—
1000 × 18.6	1	—	12/31 (39%)	7/22 (32%)	—
1000 × 27.4	1	—	0/20 (0%)	1/21 (5%)	10/35 (28%)
625 × 27.4	2	—	—	11/44 (25%)	—
1000 × 27.4	2	—	—	22/50 (44%)	17/20 (85%)
625 × 27.4(2) + 18.6(1)	3	—	0/22 (0%)	—	—

HBS = Hepes-buffered saline, final concentration 0.5 or 1.0 ×; LacZ = pRSV-lacZ; CAT = pSV2CAT; C = circular; L = linear; N = number of fry analyzed.

all 46 fry DNA samples analyzed (data not shown), showing that the fry DNA could be successfully amplified and that the PCR reactions were reliable.

Detection of pRSV-lacZ and pSV2CAT DNA in fry by Southern analysis

PCR results indicated that electroporated salmon sperm were capable of transferring plasmid DNA into salmon fry. We therefore used the DNA extracted from 16 pRSV-lacZ–positive fry and 26 pSV2CAT–positive fry for Southern hybridization, as well as DNA from 4 pRSV-lacZ–negative and 40 pSV2CAT–negative fry. None of these fry were found to be positive by Southern analysis.

Expression of the lacZ gene

Given the low levels of plasmid DNA present in the fry, it was thought unlikely that expression of the *lacZ* and *CAT* genes would be detected. However, expression of the *lacZ* gene was tested in some fry known to contain pRSV-lacZ DNA (treatment: 1,000 V/cm, 27.4 msec, 2 pulses, linear pRSV-lacZ). β -Galactosidase assays using spectrophotometric and fluorometric methods (MacGregor et al., 1991) failed to obtain evidence of *lacZ* expression. In view of these results, expression of the *CAT* gene was not assayed.

Discussion

Studies on the effect of electroporation on cell viability demonstrate a trend of decreasing survival with increasing field strengths and pulse lengths (Shigekawa and Dower, 1988; O'Hare et al., 1989; Sin et al., 1993; Weaver, 1993). However, the increase in mortality can be offset by decreasing

the pulse length (Takahashi et al., 1991; Tekle et al., 1991) and by reducing the ionic strength of the electroporation buffer (present study). Consistent with our previous observations (Sin et al., 1993), we found sperm motility was reduced in the electroporated samples when compared with the unshocked control samples. The greatest reduction of motility resulted when multiple pulses of long duration and high field strength were applied to the sperm. However, the decrease in sperm motility was lessened by using a shorter pulse. Reduced sperm motility also affected the number of embryos surviving to the fry stage, and most likely reflects a decrease in the viability and fertilizing capabilities of the electroporated sperm.

In agreement with our previous study (Sin et al., 1993), we found that electroporation enhanced transfer of plasmid DNA into salmon fry. However, some transfer of the pSV2CAT plasmid was observed without electroporation, but at a lower frequency than that obtained with the electroporated samples. The frequency of transfer of pSV2CAT by electroporated sperm was also shown to be greater than transfer of pRSV-lacZ, which could be a factor of the size of the 2 plasmids (pRSV-lacZ, 6.9 kb; pSV2CAT, 5.2 kb). However, the difference in size is small, given that mammalian cells have been successfully transfected by electroporation with DNA molecules of up to 150 kb (Shigekawa and Dower, 1988).

Transfer of pRSV-lacZ DNA was reduced at the higher field strength when the duration of the single pulse delivered to the sperm was increased. This reduction was presumably related to the increased damage to the sperm that occurred when the pulse length was extended, even though sperm

motility remained high. Thus, it appears that in this case, sperm motility cannot be used as an indicator of transfer efficiency. Nonetheless, overall there was a correlation between sperm motility and gene transfer ($r = 0.74$; $p < 0.005$); higher transfer efficiencies were achieved when the percentage of motile sperm present was reduced. Given this relationship, it was not surprising to find that transfer efficiencies were increased when 2 long pulses at the higher field strength were applied to the sperm. The reduction in sperm viability under these conditions must therefore be offset by the increased sperm/DNA association observed when 2 pulses are applied to the sperm (Symonds et al., 1994); therefore the sperm that survive have a higher probability of retaining and thus transferring plasmid DNA.

Sukharev and colleagues (1992) also found that application of a second pulse during electroporation enhanced transfer of DNA into cells, and they proposed that this enhancement was due to increased movement of DNA through the cell membrane following a second pulse. Recently, Nakanishi and Iritani (1993) also found that a double pulse enhanced DNA uptake by chicken sperm. No transfer of pRSV-lacZ DNA was detected when a triple pulse was applied to the sperm. If the magnitude or the duration of the pulse applied to cells during electroporation exceeds a critical limit, the cells are irreversibly damaged due to the loss of membrane integrity (Shigekawa and Dower, 1988; Orlowski and Mir, 1993). An increase in the damage to the sperm beyond the critical limit could explain the lack of transfer when 3 pulses were applied. Thus, for salmon sperm, as with other cell types, there appears to be a narrow range of electroporation conditions that allow efficient membrane permeabilization, DNA uptake, and an acceptable level of survival.

In our earlier study (Sin et al., 1993), we found that 625 V/cm represented a threshold field strength above which no further increase in efficiency of gene transfer was observed. This finding appears contrary to our finding herein that a higher field strength (1,000 V/cm) increases gene transfer frequencies. This difference could be due to the lower ionic strength of the buffer (0.5X HBS) used in the present study to electroporate the sperm compared with the 1X HBS buffer used by Sin and colleagues (1993). Altering the ionic strength of the electroporation buffer is known to change the optimal voltage for electroporation (Andreason and Evans, 1988). Gagne and associates (1991) also found that

buffer composition significantly affected plasmid retention by electroporated bovine sperm, and that the difference between the buffers was dependent on the strength and the duration of the electric field.

In this study we were able to improve the efficiency of DNA transfer to 30 to 85% by manipulating pulse length and field strength, pulse number, and ionic strength of the buffer. These transfer efficiencies compare favorably with other studies on fish sperm electroporation (5–10%, Sin et al., 1993; 2–4%, Müller et al., 1993), embryo baekonization (70%, Zhao et al., 1993), and embryo microinjection (75%, Chourrout et al., 1986; 50%, Guyomard et al., 1989; 25%, Culp et al., 1991). However, although we have demonstrated by PCR that sperm electroporation leads to effective transfer of plasmid DNA into salmon eggs, we were unable to detect this DNA by Southern analysis. This observation is in contrast to our previous study (Sin et al., 1993). We are currently unable to explain this discrepancy because similar protocols were used in both studies. PCR is a very sensitive technique, and it is capable of detecting less than 1 copy of plasmid DNA per 300 fish cells. These observations suggest that the fry are mosaic, and that the low levels of plasmid DNA in the fry are below the detection capabilities of Southern analysis and enzymatic detection.

The low levels of plasmid DNA in the fry may be partially due to the low plasmid DNA concentration used for electroporation. Mammalian cell electroporation studies have shown that an increase in the number of transfectants is obtained by increasing DNA concentration (Shigekawa and Dower, 1988). Thus, it may be possible to increase the amount of plasmid DNA in the developing fry by increasing the plasmid DNA concentration used for electroporation.

The high transfer efficiencies we obtained using electroporated sperm are encouraging, and given the simplicity and ease of this technique, it has the potential to be developed as a mass gene transfer method in finfish and shellfish when microinjection is not applicable. In this study, we did not examine integration of the plasmid DNA into the chinook salmon genome due to the low levels of plasmid DNA detected. However, before sperm-mediated gene transfer techniques can be used routinely to produce transgenic fish, integration of the exogenous DNA into the host genome must be achieved. Sperm-mediated gene transfer has been demonstrated in a number of farm animals, but integration of the foreign gene appears to be

unstable (Lauria and Gandolfi, 1993; Nakanishi and Iritani, 1993). We are currently investigating methods that may increase the frequency of integration.

Materials and Methods

Sperm motility assessment

Chinook salmon eggs and sperm were obtained from sea run salmon. The motility of control, buffer-treated, and electroporated sperm was determined according to the method of Sin and colleagues (1993). Sperm from a single male were used in this study.

Electroporation of sperm

Before any sperm were used for fertilization, the effect of the ionic strength of the electroporation buffer (1X HEPES-buffered saline [HBS], [pH 5.95], 21 mmol/L HEPES, 137 mmol/L NaCl, 50 mmol/L KCl, 0.7 mmol/L Na_2HPO_4 , 6 mmol/L sucrose) on sperm motility was determined. We observed that when the milt was diluted with 1X HBS at a ratio of 1:1, the motility of the sperm was more than 90%. However, when milt was diluted with 2X HBS at the same ratio, the motility was reduced to 40 to 50%. Therefore, unless otherwise stated, we used 1X HBS in all experiments.

One half mL milt (approximately 4.2×10^9 sperm) was diluted at a 1:1 ratio with 1X HBS containing 20 $\mu\text{g}/\text{mL}$ either circular or linear pRSV-lacZ (Gorman et al., 1983) or circular pSV2CAT DNA (Gorman et al., 1982) (see Figure 3). Sperm were electroporated at different field strengths (625 and 1,000 V/cm), pulse lengths (18.6 and 27.4 msec), and with a single, double, or triple pulse (see Table 1) using a Cell-Porator electroporation system (BRL), which generates pulses with exponential decay. After electroporation, sperm were incubated on ice for 10 minutes, and they were then used to fertilize 300 freshly stripped eggs. The embryos were allowed to develop to the fry stage (12 weeks) before collection.

Plasmid DNA preparation

pRSV-lacZ and pSV2CAT DNA were prepared using the alkaline lysis procedure (Sambrook et al., 1989). pRSV-lacZ was linearized by cleavage at the single *ScaI* site within the pBR322 sequence (see Figure 3).

DNA extraction

DNA was extracted from the tail region of each fry (approximately one third of the fry) using a modifi-

cation of the method of Zhao and associates (1993) to give a final volume of 70 μL in TE8 (10 mmol/L Tris-HCl [pH, 8.0], 1 mmol/L EDTA). DNA was quantified by absorbance at 260 nm and by comparison with a known amount of salmon DNA on an agarose minigel (Sambrook et al., 1989).

Detection of plasmid DNA in the fry

bPCR. PCR was used to detect pRSV-lacZ and pSV2CAT in the fry DNA samples. For circular and linear pRSV-lacZ, two 20-mer oligonucleotide primers (P322 and lacZ) were used to amplify a 1,300-bp fragment (see Figure 3). For pSV2CAT, two 20-mer oligonucleotides (Cat1 and Cat2) were used to amplify a 935-bp fragment (see Figure 3). Oligonucleotide primers were obtained from the Christchurch Medical School. As a control, a 311-bp fragment of the melanin concentrating hormone (MCH) gene of chinook salmon (Minth et al., 1989) was amplified in a separate PCR reaction from 46 randomly chosen fry DNA samples.

DNA amplification was carried out using the method of Saiki and co-workers (1988), with minor modifications. Each amplification mixture (25 μL) contained 1.0 μg fry DNA. PCR amplification was performed with a Perkin Elmer Thermal Cycler 480, and it consisted of 30 cycles of 1 minute at 94°C, 1 minute at 57°C, and 1 minute at 72°C. A final extension of 7 minutes was included. The PCR products were analyzed by 1.8% agarose gel electrophoresis, and they were transferred to Zeta-probe nylon membrane (Biorad) for hybridization analysis. For the detection of pRSV-lacZ DNA, a 160-bp fragment within the lacZ gene was amplified by PCR (see Figure 3), and it was labeled with [α - ^{32}P]-dCTP by random priming (Sambrook et al., 1989). The pSV2CAT probe was obtained by amplifying the pSV2CAT plasmid using the same primers used for the fry screening. The primer binding sites were then removed by restriction endonuclease digestion (see Figure 3), and the products were separated by agarose gel electrophoresis. The probe was excized from the gel in a trough of low melting temperature agarose, and it was used directly in a random priming [α - ^{32}P]-dCTP labeling reaction (Sambrook et al., 1989). Hybridization conditions were as described by Symonds and colleagues (1994). Membranes were exposed to radiographic film (Amersham Hyperfilm) with intensifying screens for 5 to 7 days at -80°C.

Southern analysis. For Southern blots, 15 μg undigested fry DNA were electrophoresed in a 1%

agarose gel and transferred to a Zeta-probe nylon membrane (Biorad). Membranes were hybridized to [α - 32 P]-labeled pRSV-lacZ or pSV2CAT DNA following conditions previously described (Symonds et al., 1994). Membranes were exposed to radiographic film for 3 weeks at -80°C .

LacZ gene expression assay

Following PCR analysis, some of the fry were chosen to test for *lacZ* gene expression. The remaining tissue was homogenized in 0.5 mL 0.1 mol/L sodium phosphate buffer (pH 8). The homogenate was further extracted by 3 freeze-thaw cycles using liquid nitrogen, and it was centrifuged at $10,000 \times g$ for 15 minutes at 4°C . The fry extract was assayed for β -galactosidase activity at pH 8 using the spectrophotometric and fluorometric methods as described by Sambrook and co-workers (1989) and MacGregor and associates (1991).

Acknowledgments

This study was supported by grants from the Department of Zoology, the University of Canterbury Staffing Committee, the University Grants Committee (UGC) of New Zealand, and the Foundation for Research Science and Technology (FRST) (F.Y.T.S.). J.E.S. is partially supported by a UGC postdoctoral fellowship and by FRST. The plasmids, pRSV-lacZ and pSV2CAT, were generous gifts from Dr L.A. Allison. We thank NIWA freshwater (Christchurch) for the use of their salmon hatchery at Silverstream, and L. Hawke for looking after the embryos and the fry.

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Gene transfer by electroporated chinook salmon sperm

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Received: 30 September 1994/Accepted: 8 May 1995

Abstract. Electroporation of chinook salmon (*Oncorhynchus tshawytscha*) sperm has been tested as an alternative method to microinjection for gene transfer. The plasmids pRSV-lacZ and pSV2CAT were used in this study, and the transferred DNA was detected in embryos and 12-week-old fry by the polymerase chain reaction (PCR). The effects of the following parameters on the efficiency of gene transfer were determined: field strength, pulse length, number of pulses, and the form and concentration of the DNA. Electroporation reduced sperm motility and the number of embryos surviving to the fry stage was dependent on the percentage of motile sperm used for fertilization. Plasmid DNA was detected in 96% (82/85) of the 1- to 11-day-old embryos. At 12 weeks old up to 47% of the fry retained the plasmid DNA. The highest efficiency of transfer was obtained when two pulses of 1000 V/cm and 27.4 ms were applied to the sperm incubated with 200 µg/ml of DNA.

Microinjection of DNA into newly fertilized eggs, while the most common method used to produce transgenic fish (e.g., Chen et al. 1993; Shears et al. 1991), remains problematic. Sperm and electroporated sperm have been shown to be capable of transferring DNA into embryos (Khoo et al. 1992; Müller et al. 1992, respectively). In our laboratory, we have demonstrated that electroporated chinook salmon sperm can take up plasmid DNA (Symonds et al. 1994a) and transfer this DNA into salmon embryos (Sin et al. 1993; Symonds et al. 1994b). It is hoped that sperm electroporation, once optimized, will provide a simple and quick technique for the mass production of transgenic fish.

Materials and methods

Milt (0.5 ml) was diluted in a 1:1 ratio with 1× HEPES-buffered saline containing plasmid DNA, electroporated, then incubated at 0°C for 10 min.

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Nonelectroporated sperm were similarly incubated with plasmid DNA. A small aliquot of electroporated sperm was also taken and the sperm motility was determined using the method described by Sin et al. (1993). All sperm samples were used to fertilize approximately 300 eggs. Fish of five embryonic stages (up to 11 days after fertilisation) and the fry stage were collected and stored at -80°C. DNA was extracted from embryos using the method of Chourrout et al. (1986), and from fry using the method described by Symonds et al. (1994b). The efficiency of gene transfer into embryos and fry was determined using PCR (Symonds et al. 1994b). The PCR products obtained from the fry DNA samples were analyzed by Southern hybridization using a radiolabeled probe specific to an internal portion of the plasmid PCR product (Symonds et al. 1994b).

The effects of the following variables on gene transfer efficiency were tested: the strength of the electric field (625 V/cm and 1000 V/cm), the length of time this field was applied to the sperm (pulse length, 18.6 and 27.4 ms), the number of pulses (0, 1, and 2), the type of DNA incubated with the sperm (i.e., different plasmids, pRSVlac-Z and pSV2CAT), the form of the DNA (circular and linear), and the concentration of the DNA (20, 100, and 200 µg/ml).

Results and discussion

Motility of sperm after electroporation

Sperm motility was found to decrease with increasing field strength, pulse length, and number of pulses. When two pulses, each of 27.4 ms at 1000 V/cm, were applied, less than 5% of the sperm remained motile. Other studies on the effect of electroporation on cell viability have also demonstrated a trend of decreasing survival with increasing field strength and pulse lengths (Shigekawa and Dower 1988; O'Hare et al. 1989). The number of eggs fertilized and surviving to the fry stage decreased when two pulses were applied to the sperm, and it was shown to be dependent on the percentage of motile sperm present.

Detection of pRSV-lacZ DNA in embryos using PCR

To determine the amount and fate of the exogenous plasmid DNA, individuals from five early embryonic stages were analyzed up to 11 days after-fertilisation (stage 14, mid-gastrulation). Initial results have shown that all sperm mixed with circular pRSVlac-Z DNA (100 and 200 µg/ml) were capable of transferring this DNA into a high proportion (82 of 85) of embryos, irrespective of the electroporation conditions used (no pulse, 1 pulse of 18.6 ms or 2 pulses of 27.4

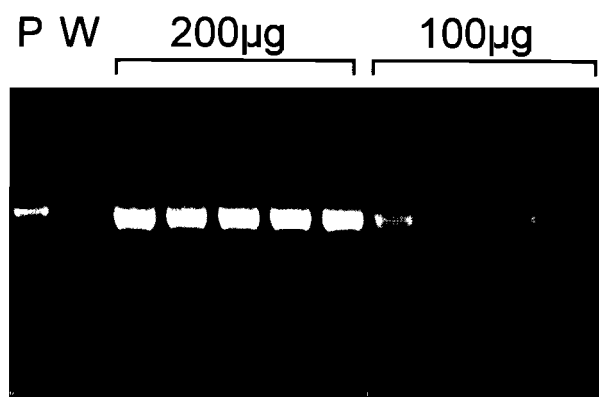


Fig. 1. Detection of plasmid DNA in late blastula stage (80 h after fertilization) salmon embryos using PCR. The effect of circular pRSV-lacZ DNA concentration (100 or 200 µg/ml) on the amount of plasmid specific PCR product obtained is shown. P = positive control (1.3 kb); W = water control. The electroporation conditions for all samples were two pulses of 1000 V/cm \times 27.4 ms.

ms). The amount of product resulting from PCR amplification of the embryo DNA samples varied and was dependent on the electroporation conditions, the concentration of the plasmid DNA, and the age of the embryos analyzed. Increasing amounts of PCR product were obtained from the 0-, 1-, and 2-pulse treatments, respectively (data not shown), and more product was obtained when the plasmid DNA concentration was increased from 100 µg/ml to 200 µg/ml (Fig. 1). The amount of plasmid-specific PCR product decreased with increasing age of the embryos (data not shown), although over 95% of the oldest embryos still contained a detectable amount of plasmid DNA.

Detection of pRSV-lacZ and pSV2CAT DNA in fry using PCR

1992 salmon breeding season. Fry from 18 different treatments were collected and the DNA from a sample of each of these was analyzed by PCR (Symonds et al. 1994b). A high frequency of plasmid DNA transfer was observed only when electroporated sperm were used to fertilize salmon eggs. Transfer efficiencies of 0–85% were obtained. The frequency of transfer was affected by the electroporation conditions and the type of plasmid used (pSV2CAT gave a higher frequency than pRSV-lacZ). The highest transfer ef-

ficiency was obtained for both plasmids when a double pulse of 1000 V/cm and 27.4 ms was applied to the sperm. Sukharev et al. (1992) found that the application of a second pulse during electroporation enhanced transfer of DNA into cells. Two consecutive pulses is also known to enhance DNA uptake by chicken sperm (Nakanishi and Iritani 1993). This is probably due to increased movement of DNA through the cell membrane following a second pulse (Sukharev et al. 1992).

1993 salmon breeding season. The effect of increasing the concentration of the DNA incubated with the sperm was tested using the most successful electroporation conditions as determined in 1992 (1 pulse of 18.6 ms \times 1000 V/cm and 2 pulses of 27.4 ms \times 1000 V/cm).

Preliminary analysis has been carried out (Table 1). Although gene transfer efficiencies were variable, some conclusions can be drawn from the results. Electroporated sperm were more efficient at transferring DNA into fry, and transfer efficiencies of 0–47% were obtained. In seven of eight treatments where the application of a single (1 \times 18.6 ms) or a double (2 \times 27.4 ms) pulse was analyzed, the double-pulse treatment gave a higher gene transfer efficiency. In addition, the highest DNA concentration (200 µg/ml) produced the highest gene transfer frequency (47%).

Overall the pattern of gene transfer efficiencies obtained for the two plasmids used was different. In agreement with the results from the embryo PCR analysis, increasing the pSV2CAT DNA concentration resulted in a higher gene transfer frequency. However, this trend was not observed for pRSV-lacZ, where the highest transfer efficiency was obtained with the lowest DNA concentration.

The PCR analysis of the salmon embryos indicates that, although present in a large proportion of individuals, most of the introduced plasmid DNA remained unintegrated and had undergone extrachromosomal amplification early on in development. However, by the time the fish reached the fry stage, the amount of exogenous DNA had decreased to low levels. No plasmid DNA was detected in fry DNA samples from the 1992 season by Southern hybridization analysis (Symonds et al. 1994b). These results suggest that the fry are mosaic. This pattern is analogous to the fate of plasmid DNA microinjected into one or two cell embryos of zebrafish, medaka, loach, and African catfish (Stuart et al.

Table 1. Effects of DNA concentration and electroporation conditions on efficiency of gene transfer (percentage) by salmon sperm.^a

DNA (µg/ml)	No. of pulses	Field strength (V/cm) \times pulse length (ms)	LacZ,C (%)	LacZ,L (%)	CAT,C (%)	CAT,L (%)
100	0				6.7	
200	0		0	0	6.7	7
20	1	1000 \times 18.6	0	20	3.6	
100	1	1000 \times 18.6	3.3	0	6.7	
200	1	1000 \times 18.6	10		13.3	36.7
20	2	1000 \times 27.4	33.3	12.5	16.7	
100	2	1000 \times 27.4	14.8	6.9		
200	2	1000 \times 27.4	25	6.7	33.3	46.7

^aLacZ = pRSV-lacZ; CAT = pSV2CAT; C = circular; L = Linear. Sample sizes range from 20 to 30.

1988; Chong and Vielkind 1989; Kozlov et al. 1989; Volckaert et al. 1994). Therefore, plasmid DNA introduced into embryos by electroporated sperm appears to behave similarly to DNA transferred directly by microinjection.

In conclusion, electroporated sperm are capable of fertilizing eggs and transferring plasmid DNA into salmon embryos. The efficiency of gene transfer is dependent on the electroporation conditions, and transfer efficiencies of over 40% can be achieved repeatedly. Overall, the optimal condition appears to be two 1000 V/cm pulses of 27.4 ms with 200 µg/ml of DNA. However, the precise conditions may vary between gene constructs.

Acknowledgments. Financial support through the Foundation for Research Science and Technology, NZ, is gratefully acknowledged. We thank L. Hawke (NIWA) for rearing the embryos and fry.

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